Capillary Electrophoresis for Protein Analysis

Strategies to Prevent Protein Adsorption and Method Development Using Coated Capillaries for Electrophoresis

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Dedications

to:

My Parents,

Husband, Son and Brother

List of Abbreviations

abc	4-amino benzoic acid
Bicine	N,N-bis(2-hydroxyethyl)glycine
BGE	background electrolyte
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CD	cyclodextrine
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CZE	capillary zone electrophoresis
DDAB	N,N-didodecyl-N,N-dimethylammonium bromide
DEGMA	diethylene glycol monomethacrylate
DMAA	*N*,*N*-dimethylacrylamide
DMSO	dimethylsulfoxide
EOF	electroosmosis flow
EPDMA	epoxy-poly(dimethylacrylamide)
ESI-MS	electrospray ionization-mass spectrometry
FSCE	free solution capillary electrophoresis
GC	gas chromatography
HEC	hydroxyethylcellulose
HEMA	hydroxyethyl methacrylate
HMPA	hexamethyl phosphoric acid triamide
HPLC	high performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
ID	internal diameter
LC	liquid chromatography
LPA	linear polyacrylamide
MALDI-TOF-MS	matrix-assisted laser desorption/ionization-time-of-flight mass spectometry

MEKC	micellar electrokinetic chromatography
MPS	methacryloyloxypropyl trimethoxy silane
MS	mass spectrometry
PB-PVS	polybrene and poly(vinyl sulfonate)
PDMAA	poly(dimethylacrylamide)
PEG	poly(ethylene glycol)
PHEA	Poly-N-hydroxyethylacrylamide
p*I*	isoelectric point
PVA	poly(vinyl alcohol)
RE-CZE	reversed-charge Capillary Zone Electrophoresis
RSD	relative standard deviation
SAM	self-assembled monolayers
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEGMA	triethylene glycol monomethacrylate
Trp	tryptophan
UV	ultraviolet
VIS	visible
WCID	whole-column imaging detection

List of Symbol

κ	molar extinction coefficient or absorptivity [$M^{-1}cm^{-1}$]
η	viscosity of the solution [Pa.s = $N.s.cm^{-2}$]
ρ	density of the solution [$kg.m^{-3}$]
μ_a	apparent electrophoretic mobility
μ_i	electrophoretic mobility [$m^2V^{-1}s^{-1}$]
μ_{eof}	EOF mobility [$m^2V^{-1}s^{-1}$]
$\mu_{EOF\ app}$	apparent EOF mobility [$m^2V^{-1}s^{-1}$]
ε	dielectric constant
ζ	zeta potential
v	ion migration velocity [$m.s^{-1}$]
$v_{i(net)}$	net velocity of component i [$m.s^{-1}$]
v_{eof}	electroosmotic flow velocity [$m.s^{-1}$]
v_i^0	migration velocity of component i [$m.s^{-1}$]
z_i	charge number of component i
e_0	elemental charge [$1.602.10^{-19}$ C]
A	peak area [AU.min]
c_i	concentration of component i [M]
d	optical path length of light through the detection cell [cm]
E	electric field strength [Vm^{-1}]
$E(\lambda)$	adsorbed light intensity
F_e	electric force [N]
F_d	drag forces [N]
g	gravitational acceleration ($9.80665\ N.kg^{-1}$)
Δh	height difference [m]
I	electric current [A]
I_0	initial light intensity
I_t	light intensity after absorbance
L_D	capillary length to detector or effective capillary length [cm]
L_T	distance between the electrodes or total capillary length [cm]

P_{inj}	injection pressure [mbar]
Δp	pressure difference [Pa]
P_{add}	additional pressure [mbar]
q	charge on the ion [C]
Q_i	amount of species i
r	inner radius of capillary [m]
r_i	ion radius
R	electric resistance of the electrolyte [$V.A^{-1} = \Omega$]
t_i	migration time of component i [s]
t_{eof}	migration time of EOF marker [s]
t_{inj}	injection time [s]
t_{mig}	migration time [min]
U	voltage [V]
V_i	injection volume [m^3]

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1. Introduction

1.1. General aspects of capillary electrophoresis

Capillary electrophoresis (CE) is described as high-efficiency separations techniques that use narrow-bore fused-silica capillaries to separate large and small molecules. These separations of molecules are based on differences in charge, size and hydrophobicity in high electric field strengths. Depending on the types of capillary and electrolytes used, the technology of CE can be divided into several separation techniques. For each of them, many strategies for method development were evolved to achieve selectivity, efficiency, precision, short analysis time, reduced sample pre-treatment requirements and validation.

1.1.1. Applicability of CE

1.1.1.1. Application of CE

CE is applicable for a wide range of compounds, from small to large molecules for instance nucleotides and proteins [1, 2]. Analysis using CE has evolved into an irreplaceable tool for the quality control of pharmaceuticals and biotechnological products. In other cases CE is established as alternative technique in pharmaceuticals routine analysis. Application in pharmaceuticals field is specifically for the determination of drug-related impurities, drug potency, chiral analysis, and determination of drug counterion content. For these application, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) are frequently used [3].

CE has increasingly been used for peptide and protein analysis, because it is reliable, versatile and productive. There are many alternative CE separation modes that are applicable to peptide and proteins including CZE, MEKC, CIEF (capillary isoelectric focusing) and CITP (capillary isotachophoresis). The matured CE has become a superb complement to HPLC, in many cases has also evolved as an automated and quantitative replacement for conventional slab gel electrophoresis methods such as SDS-PAGE and isoelectric focusing [4].

Analysis of nucleotides using CZE, MEKC, and recently, routinely-permits high-resolution separations of oligonucleotides using capillary gel electrophoresis (CGE) were frequently reported [3, 5].

It has been usually reported, that the application of chiral separation is mostly performed by CE. Separations have been achieved in free-solution capillary-electrophoresis (FSCE) employing cyclodextrines (CDs) as chiral selectors and also in MEKC through the use of chirally selective micelles [3].

The other application areas of CE are for determination of small ions, amino acid, carbohydrate, vitamin, agrochemicals, biomedical and large polymer species [3, 5]. Nowadays, fabrication of micro CE chips for chemical and biomedical applications has been widely investigated. Some successful separations of protein samples using micro CE chips have also been frequently reported [4, 5].

1.1.1.2. Advantages of CE

CE has several general advantages compared to other analytical separation techniques, such as high separation efficiency, speed of analysis, flexibility, precision, simplicity and economical in terms of labor, solvent volumes, waste disposal, stationary phases, nanoliter sample amounts possible, and little or no sample pretreatment necessary. These advantages of CE have turned this technique into a well established alternative to liquid chromatography (LC) [2, 3, 6].

CE provides greater resolution of a larger number of peptide fragments and benefits in the analysis of large protein. The separation of protein and DNA was achieved using gel-filled capillaries, which easier and more accurate quantification of the peaks compare to traditional one-dimensional gel electrophoresis [3].

Based on the separation efficiency, high selectivity and lower costs, CE is the preferable technique in pharmaceutical quality control compared to HPLC. However, the precision in both techniques are equal [6]. During recent years, it has been shown that CE is an excellent technique for resolution and quantitation of enantiomers. The main advantages of this technique are high efficiency, fast analysis time and possibility of using new selectors [3].

Some samples containing complex matrix components (plasma samples, polymer solutions, plant extracts, etc.) can be directly injected without any further pre-treatment. CE capillaries can be easily cleaned and replaced, and therefore, they are cheaper compared to GC or HPLC columns. In the separation using micro capillary electrophoresis chips, analysis times in the μs range and extremely high sample number are possible. Good quantitative data (relative

standard deviation; RSD% [peak area]: 1%) are easily obtained by analyte concentrations above 100 mg/L, but RSD% [peak area] of 2-5% could still be achieved in concentration of about 10 mg/L [6].

1.1.1.3. Techniques of CE

The main modes of CE that have been developed include capillary zone electrophoresis (CZE), often referred to as free-solution CE (FSCE) that is based on differences in the charge and size of the analyte; micellar electrokinetic capillary chromatography (MEKC) in which the compound is separated using surfactant micelles; capillary isoelectric focusing (CIEF) that allows amphoteric molecules to be separated in a pH gradient; capillary gel electrophoresis (CGE) that using various types of sieving media to separate sample components; and capillary isotachophoresis (CITP) in which sample components migrate between leading and terminating electrolytes [2, 3, 5].

1.1.2. Capillary electrophoresis system

1.1.2.1. General aspects

CE is described as high-efficiency separation techniques using narrow-bore fused-silica capillaries (normally 25 to 100 μm in internal diameter (ID)) to separate a complex of large and small molecules. High electric field strengths are used to facilitate this separation based on differences in charge, size and hydrophobicity. The movement or migration of charged ions in an electric field define the process of electrophoresis. A CE instrument is generally composed of an autosampler, a detection module, a high-voltage power supply, the capillary and a computer to control the separation (Figure 1).

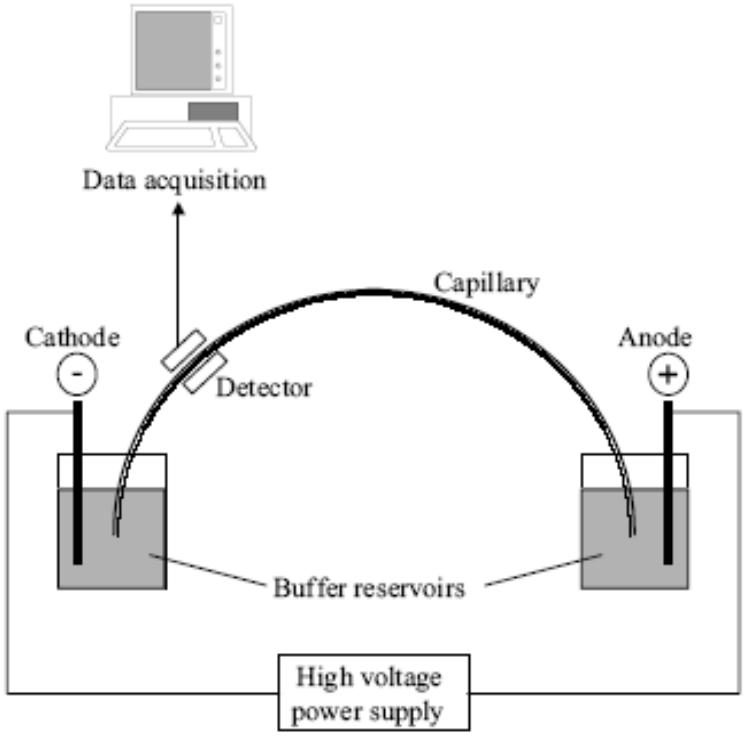


Fig. 1. A schematic instrument for CE [7]

Principally, both ends of a capillary are placed in separate buffer reservoirs, each containing a positive (anode) and negative (cathode) electrode. Sample injection is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. Application of a high voltage (normally performed in the region of 5-30 kV) causes electrophoretic and electroosmotic movements through the capillary. Consequently, ions of different charge in the sample move through the solution towards the electrode of opposite charge. Optical (UV-VIS or fluorometric) detection of separate analytes can be achieved directly through the capillary wall near the opposite end. The data output is presented plotting detector response against migration time in the form of an electropherogram [2, 3, 5, 7].

1.1.2.2. UV-detection

As standard, CE instruments use UV absorbance detectors. However, a few instruments also offer the possibility of diode array, fluorescence or laser-induced fluorescence detection. The most important requirements for the design of detectors suitable for CE systems are: small volume detection cell, small contribution to the peak width, high sensitivity, large dynamic

range, fast detector response, good resistance against temperature changes, reliable and convenient ease of use.

In general, UV-VIS detection produces rather low sensitivity compared to other detection modes developed for capillary separation systems, such as electrochemical, mass spectrometric and fluorimetric detection. However, because of its sufficient sensitivity to a wide range of compounds and functional groups, it is still the most popular detector. For quantification, Lambert-Beer's law describes the intensity of absorbed light at the wavelength λ in dependence of the concentration c [M] of the analyte and of the optical path length of light through the detection cell d [cm] (Eq. 1).

$$E(\lambda) = \log \frac{I_0}{I_t} = \kappa \cdot c \cdot d \qquad \text{(Eq. 1)}$$

$E(\lambda)$ = adsorbed light intensity
I_0 = initial light intensity
I_t = light intensity after absorbance
κ = molar extinction coefficient or absorptivity [$M^{-1}cm^{-1}$] [5]

1.1.2.3. Capillary

Capillaries used in CE consist of fused silica (amorphous SiO_2) with typical dimensions 10 to 100 cm long and an inner diameter between 25 and 100 µm. Very high voltages can be applied using these narrow-bored capillaries [2, 3, 6]. As fused silica coating is susceptible to abrasive damage and subsequent breakage, it is necessary to protect the outer surface with polyimide. This coating strongly absorbs UV light and it is, therefore, necessary to remove the coating in the area of the capillary used as the window for on-column detection [3].

Although separation can be performed on fused-silica capillaries, most manufactured capillaries are highly variable in quality and less than optimal for application. Some problems in the capillary manufacturing techniques are variations in capillary bore and outer dimensions, ovality, random brittleness, and surface activity [8]. In the same capillary, a wide range for surface roughness values has been found. These variations can be caused by sample-preparation artefacts and local surface defects, e.g. caused by storage. Bulged structures are frequently found which are probably crystallization products of carbonates built up during storage [9].

Chemical modifications of the capillary wall in electrophoresis have been frequently reported. Many reasons for the capillary wall modification include reduction or elimination of analyte-wall interactions, alteration of the electroosmotic flow (EOF) to produce a more rapid separation, improved reproducibility or resolution, especially for difficult separation. Particularly for proteins or larger biomolecules which tend to adsorb strongly to the silica, the separation efficiencies can only be achieved if the analyte-wall interaction is carefully suppressed through changes in charging and adsorptivity of the fused-silica surface by chemical modification on the capillary wall. The capillary wall coating is ideally homogene, stable under conditions required for separation, preferably over a broad range of buffer pH, and allows a reproducible application of the coating [10]. There are several ways to coat a fused silica tube, including:

1.1.2.3.1. Dynamic coating capillary

By this way, the capillary surface can be coated dynamically by employing additives such as surfactants, zwitterionic salts or hydrophilic linear polymers to the buffer system. This procedure is advantageous because of its simplicity and low costs. However, it also provides several drawbacks, i.e. reproducible dynamic coating is difficult to achieve, changes in the buffer composition alter the coating condition, and disturbing interactions with the analytes may occur, several proteins precipitating in the presence of ionic surfactants [5].

The formation of dynamic coating occurs in equilibria between the buffer and the capillary surface. Coatings cover the surface and the charges on it which afterwards can prevent the formation of the double layer that gives rise to the EOF. The effectivity and stability of coatings depend mainly on the energy of intermolecular interaction or adsorption of the modifying molecule at the surface and on the concentration of the modifying additive in the buffer [11].

Dynamic wall coating is prepared by rinsing the capillary with a solution containing a coating agent. A polymer or a small molecular-mass compound is typically used as coating agent. Since the attachment of the coating to the wall is based on adsorption, a small amount of coating agent is usually added to the separation medium to keep the coating on the capillary wall surface. An occasional regeneration is also required because of the limited lifetime of a dynamic coating capillary. It is attractive because of its ease of preparation, but more work is

needed to assure the required capillary maintenance such as regeneration, washing between runs, and other recursive steps [10].

Many types of polymeric and small molecular mass buffer additives are used as dynamic coatings, for example: PB-PVS (polybrene and poly(vinyl sulfonate)) for analysis of protein and peptide [12, 13], DDAB (N,N-didodecyl-N,N-dimethylammonium bromide) for separation of basic proteins [14], PHEA (Poly-N-hydroxyethylacrylamide) for application in DNA and protein separation [15, 16], Poly(HEMA) (poly(hydroxyethyl methacrylate)), poly(DEGMA) (poly(diethylene glycol monomethacrylate)), poly(TEGMA) (poly(triethylene glycol monomethacrylate)) for protein analysis [17]. Triton X-100, Brij 35, Tween 20, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) as surfactants are investigated in preventing protein binding to the fused silica surface [18].

Verzola (2000) investigated 4 polymers, such as HPMC (hydroxypropylmethylcellulose), HEC (hydroxyethylcellulose), PVA (poly(vinyl alcohol)) and poly(DMAA) (poly(dimethylacrylamide)) to inhibit the adsorption of protein to silica wall. HPMC, PVA, HEC could inhibit adsorption only by, at most, 50% [19].

1.1.2.3.2. Permanent coating capillary

An alternative strategy to reduce adsorption is to bond chemically a polymer to the capillary surface or to modify the active sites of the silica by derivatization. If a polymer is used for coating, it is anchored to the silica by reaction of only a part of silanol groups with a reagent. In contrast, if the surface is derivatized, the coating is only effective if all active silanol groups react with the reagent. A chemical capillary coating should be effective in suppressing adsorption, allow a constant electroosmotic flow over a wide range of pH, be reproducible in preparation, be stable for a long time, and stable over a wide range of pH [5].

A permanent wall coating is considered as an attractive way to eliminate the EOF and wall-analyte interaction in the separation capillary. The capillary performance deteriorates during repetitive runs and extensive rinsing is required between the runs. Covalently derivatized capillaries still exhibit longer lifetimes and require less maintenance than dynamic coated ones, but the reliable preparation of these coatings can be challenging. Based on their separation performance, polyacrylamid-coated capillaries are still superior to any other type of coating. Three steps for preparation of a permanent wall-coating are, respectively, capillary

pre-treatment, introduction of double bonds to the capillary wall, and binding of a polymer to this intermediate layer [10].

EPDMA (epoxy-poly(dimethylacrylamide)) [20] and linear polyacrylamide have been used to eliminate wall interactions with proteins [21]. Dimethylacrylamide as a permanent coating also shows stability for the separation of proteins and peptides [22].

The practical aim in generating such coatings must be to form surface layers that are stable and not disrupted during long series of measurements even with chemically aggressive buffers, i.e., in the wide range of buffer pHs. Moreover, permanent coatings must not be destroyed by rinsing procedures with any of the aggressive rinsing solutions that might be applied for regeneration of the separation performance as determined by the status of the capillary surface [11].

Before the coating solution is introduced into the capillary, the silica surface must be cleaned and activated. This reaction is shown in Figure 2.

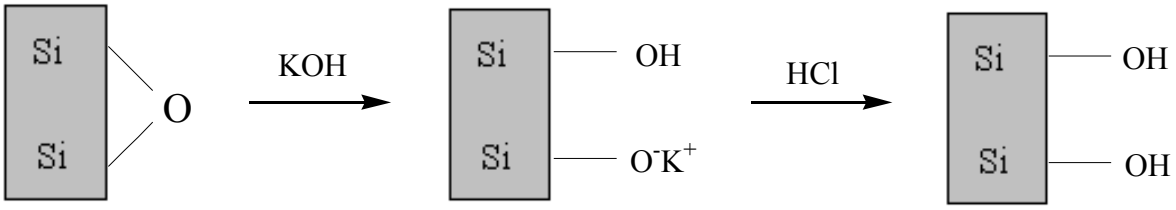


Fig. 2. Reaction for surface activated [5]

Hjertén (1985) described a polyacrylamide coating that is generated by polymerization on a surface previously silanized with the methacryloxy-propyl-trimethoxysilane reagent [21]. The reaction to obtain polyacrylamide coating is shown in Figure 3.

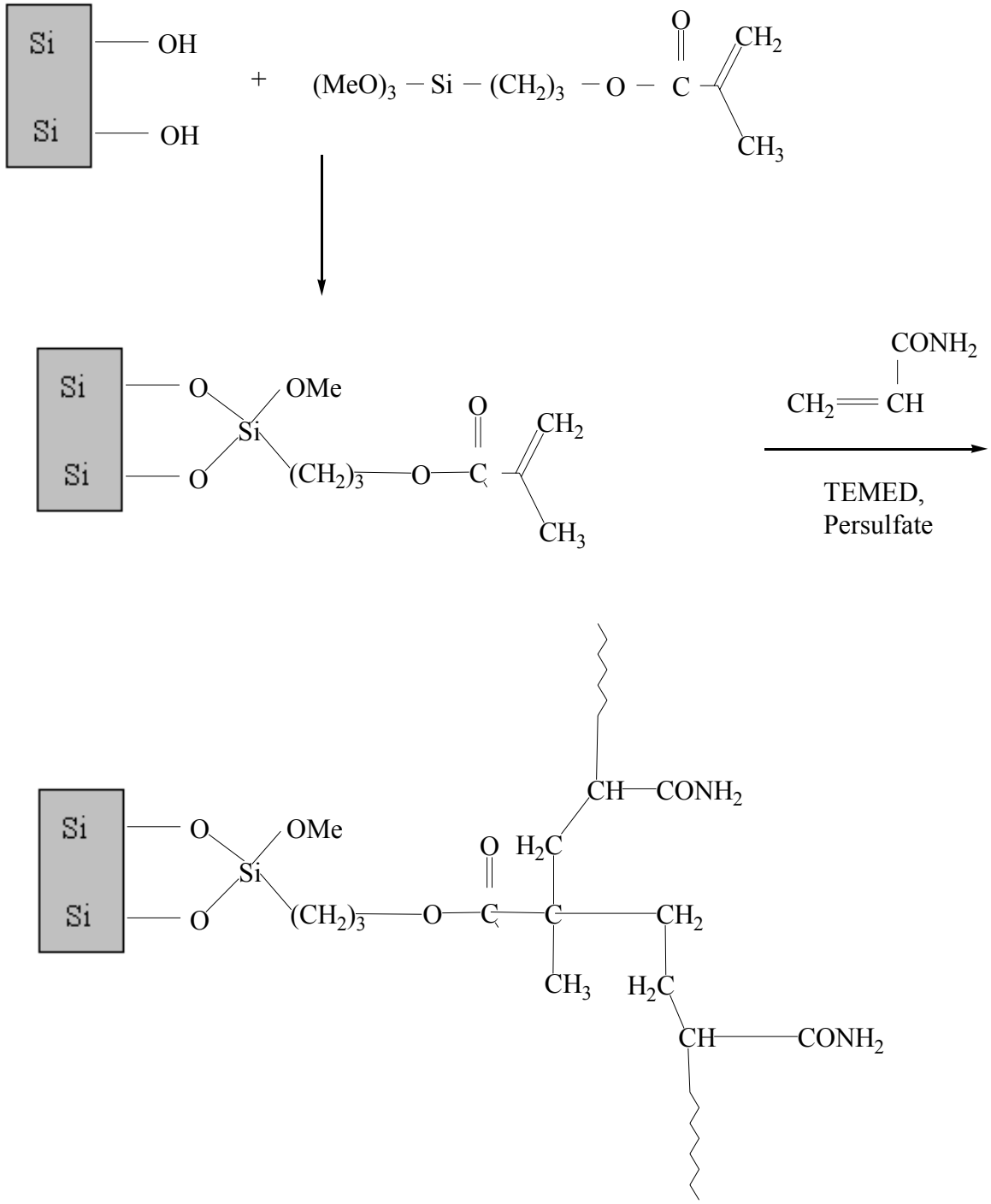


Fig. 3. Reaction for coating of silica with polyacrylamide by silanization with methacryloxy-propyl-trimethoxysilane [5]

Another reaction is based on the formation of a vinylated silica surface by a Grignard reaction of clorinated silanols (using $SOCl_2$) with $BrMgCHCH_2$ and the vinyl group is bonded via SiC. The reaction is shown in Figure 4.

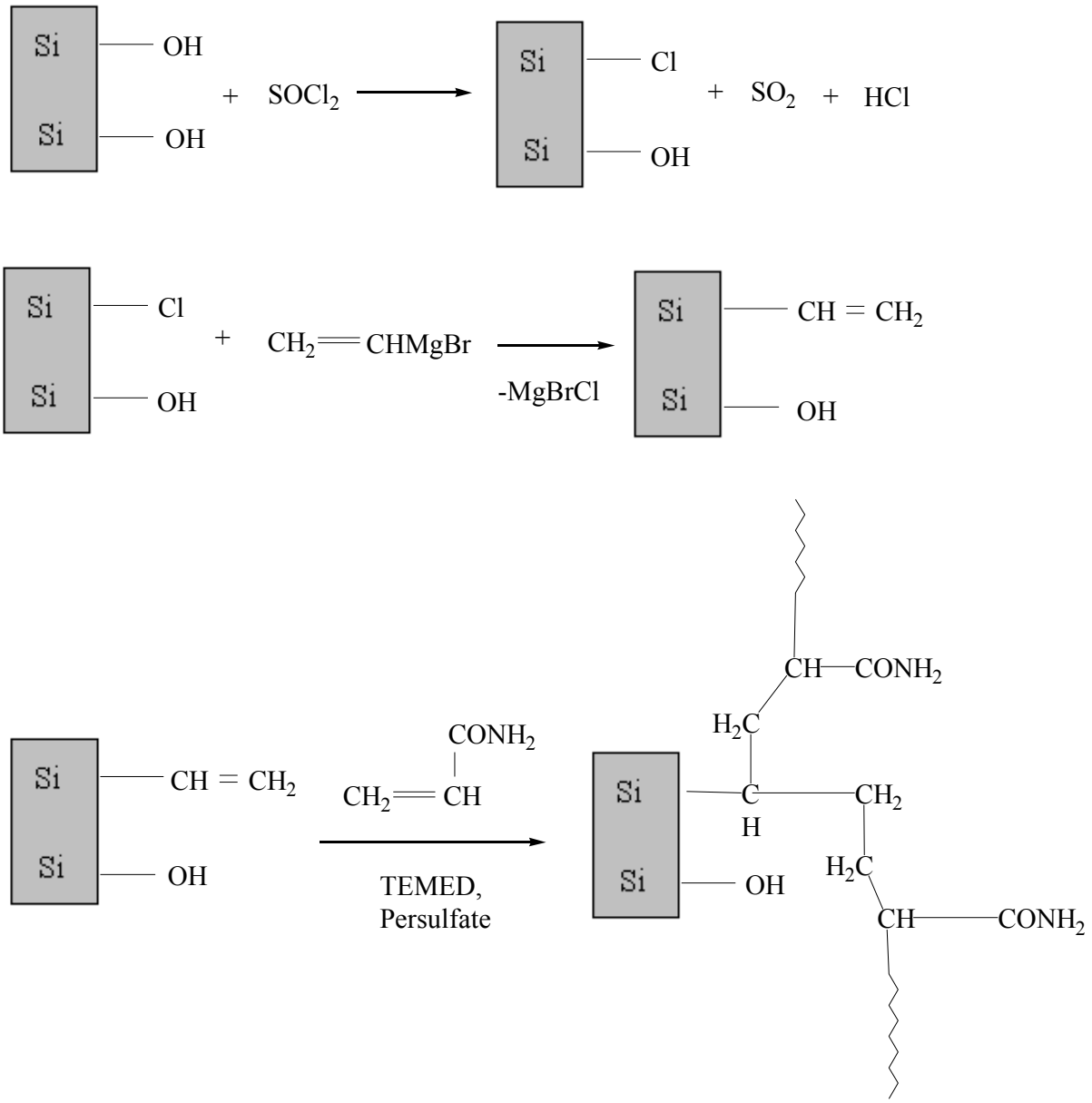


Fig. 4. Reaction for vinyl-bound polyacrylamide coating [5]

1.1.3. Sample injection

Sample injection is a crucial procedure in capillary electrophoresis. Only small amounts of liquid are brought into the capillary, and the repeatability of the sample volume must be high. Using an optimized electrophoretic system, sample injection has been performed carefully to produce a satisfactory separation. The sample can be introduced into the capillary by two fundamental ways that involve immersing the capillary end into the sample vial and applying a force to inject the sample into the capillary.

1.1.3.1. Hydrodynamic injection

In this part, a pressure drop has to be applied along the capillary either by high pressure at the injection side, vacuum at the detector side, or hydrostatic pressure by utilizing gravity. By high pressure at the injection side, the capillary is immersed into the sample solution, and then a pressure difference is applied to allow the sample to be introduced into the capillary. Alternatively a vacuum is applied at the end of the capillary to suck up the solution into the tube. After the sample injection has been completed, the separation process is performed by the replacing the end of the capillary into the buffer vial.

In principle, the hydrodynamic injection volume introduced into a capillary is a linear function of the applied pressure difference along the capillary and its injection time. The volume of sample solution injected into circular tube can be calculated by Poiseuille's law,

$$V_i = \frac{\Delta p \cdot \pi \cdot r^4 \cdot t}{8 \cdot \eta \cdot L_T} \qquad \text{(Eq. 2)}$$

V_i = injection volume [m^3]

Δp = pressure difference [Pa]

r = inner radius of the capillary [m]

t = injection time [s]

η = viscosity [Pa.s]

L_T = total capillary length [m]

If sample introduction is accomplished by gravity injection, the volume injected is defined as:

$$V_i = \frac{\rho \cdot g \cdot \Delta h \cdot \pi \cdot r^4 \cdot t}{8 \cdot \eta \cdot L_T} \qquad \text{(Eq. 3)}$$

ρ = density of the sample solution [$kg.m^{-3}$]

g = gravitational acceleration (9.80665 $N.kg^{-1}$)

Δh = height difference between liquid levels of samples and buffer vials [m]

As shown at Eq. 2, the sample volume introduced into the capillary can be controlled by varying the injection time and/or the pressure difference. Temperature has also an influence on injection volume because changing the temperature causes a change in the viscosity of solution. Therefore, it is important to use a constant temperature to produce reproducible injection volume. In general, the type of hydrodynamic injection produces quantitative and reproducible results. [5]

1.1.3.2. Electrokinetic injection

Electrokinetic injection offers an alternative technique for sample introduction in capillary electrophoresis. Using this technique, the capillary and the electrode are immersed into the sample solution and high voltage is applied. Principally, voltage causes electrophoretic and electroosmotic movement. Hence when high voltage is applied for a short time interval, sample is introduced into the capillary due to electrophoretic migration. Then additionally, a sample volume will be introduced into the column due to electroosmotic flow. The injected sample volume is then given by

$$V_i = v_{eof} \cdot \pi \cdot r^2 \cdot t \qquad \text{(Eq. 4)}$$

v_{eof} = electroosmotic flow velocity [m.s^{-1}]

The quantity of a species *i* introduced into the capillary during electrokinetic injection is related to various factors (Eq. 5).

$$Q_i = \frac{(\mu_i + \mu_{eof}) \cdot \pi \cdot r^2 \cdot U \cdot c_i \cdot t}{L_T} \qquad \text{(Eq. 5)}$$

Q_i = amount of species *i*

μ_i = electrophoretic mobility [m^2.V^{-1}.s^{-1}]

μ_{eof}= electroosmotic mobility [m^2.V^{-1}.s^{-1}]

U = voltage [V]

c_i = concentration of species *I* [M]

The quantity of sample introduced into the capillary can be controlled by varying voltage and/or injection time. Furthermore, it is influenced also by the electrophoretic and electroosmotic mobility of component. While hydrodynamic injections provide quantitative and more reproducible of results, electrokinetic injections produce sharp, well resolved peaks and provide more sensitivity [5].

1.2. Capillary zone electrophoresis

Capillary zone electrophoresis also known as free solution capillary electrophoresis is the simplest form of CE and the most commonly utilized. The separation mechanism of this technique is based on the difference of size and charge of analytes. The component mixture of

cationic, neutral and anionic solutes that is introduced into the capillary is separated under an application of high voltage (Figure 5).

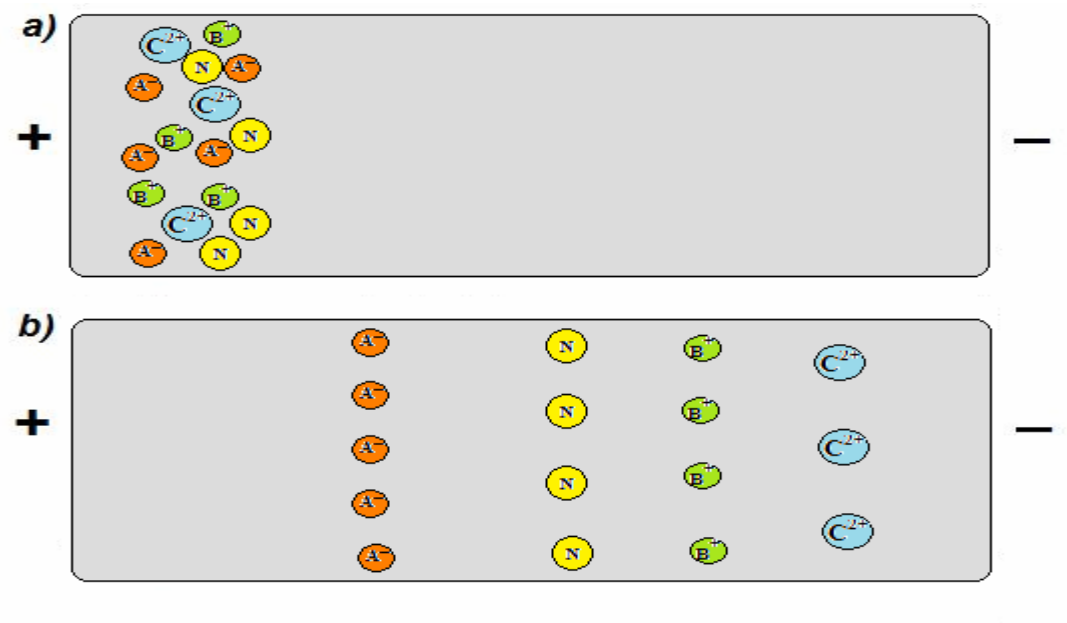


Fig. 5. Sample separations in CZE

1.2.1. Electrophoretic mobility

The electrophoretic mobility describes the movement or migration of ions through a medium (such as a buffer solution) under the influence of an applied voltage. Therefore, the separation process depends on the differences in the migration speed of ions that can be expressed as in Eq. 6.

$$v = \mu_i \cdot E \qquad \text{(Eq. 6)}$$

v = ion migration velocity [ms^{-1}]

μ_i = electrophoretic mobility [$m^2V^{-1}s^{-1}$]

E = electric field strength [Vm^{-1}].

The velocity difference of ion migration depends on the difference of charge and size of ions. In principle, the equation of electrophoretic mobility is:

$$\mu_i = \frac{q}{6 \cdot \pi \cdot \eta \cdot r_i} \qquad \text{(Eq. 7)}$$

q = charge on the ion [C]

η = solution viscosity [Pa.s]

r_i = ion radius [m]

According to Eq. 7, the electrophoretic mobility of any given ion is specific and constant. Therefore, the different ions and solutes have different electrophoretic mobility that causes a possibility to separate mixtures of different ions and solutes by using electrophoresis.

In the electrophoretic separation techniques, an analyte behaves as solvated particle in an electrolyte solution that is obtained by electrical forces along an electrical field gradient. The electric current in the solution under the influence of an applied voltage follows Ohm's law (Eq. 8).

$$U = R \cdot I \qquad \text{(Eq. 8)}$$

U = voltage [V]

R = electric resistance of the electrolyte [Ω]

I = electric current [A]

In an homogenous electric field, the charged component *i* is accelerated by the electric force as shown in Eq. 9.

$$F_e = z_i \cdot e_0 \cdot E \qquad \text{(Eq. 9)}$$

F_e = electric force [N]

z_i = charge number of component *i*

e_0 = elemental charge [$1.602.10^{-19}$ C]

E = electric field strength [$V.cm^{-1}$]

Nevertheless, the drag forces in a viscous hydrodynamic medium is needed to drive the charged component *i* against the electric strength. It is proportional to the migration velocity v_i^0 and to the Newtonian viscosity η of the medium. For spherical ions according to Stokes's law, the constant *k* can be substituted by $6 \pi r$ (Eq. 10).

$$F_d = k \cdot \eta \cdot v_i^o = 6 \cdot \pi \cdot r_i \cdot \eta \cdot v_i^o \qquad \text{(Eq. 10)}$$

F_d = drag forces [N]

k = constant [cm]

η = Newtonian viscosity of the solution [$N.s.cm^{-2}$]

v_i^0 = migration velocity of component *i* [$cm.s^{-1}$]

During electrophoresis, a steady state is achieved in which the electric force is counterbalanced by the drag force. In this condition, the charged component *i* moves with a constant migration velocity (Eq. 11) [5].

$$v_i^0 = \frac{z_i \cdot e_0}{6 \cdot \pi \cdot \eta \cdot r_i} \cdot E \qquad \text{(Eq. 11)}$$

1.2.2. Electroosmotic mobility

From Eq. 11, the proportionality factor of migration velocity to electric field strength is expressed as the absolute electrophoretic mobility (μ_i^0), as shown in Eq. 12.

$$\mu_i^0 = \frac{v_i^0}{E} = \frac{z_i \cdot e_0}{6 \cdot \pi \cdot \eta \cdot r_i} \qquad \text{(Eq. 12)}$$

Electroosmosis or electroendoosmosis is a fundamental processes in electrophoretic separation processes. This process is the movement of the bulk flow of liquid through the capillary relative to the charged surface which is caused by an electric field. This movement is also called electroosmotic flow (EOF). The EOF depends on the composition of the capillary and the nature of the solution.

As mentioned before, the fused-silica capillary is typically used for separations in CE. The surface of this capillary provides ionisable silanol groups, which are in contact with the buffer within the capillary. Under aqueous conditions (at pH value above 2.5), these silanol groups readily dissociate and then give the capillary wall a negative charge. When the capillary is filled with buffer, the positively charged ions of the buffer will be electrostatically attracted to the negatively charged capillary wall. There will be formed an electric double layer and the potential difference known as zeta potential, which takes place very close to capillary wall. The layer closest to the capillary wall is the immobile layer as described according to Stern's model in Figure 6. Stern's model includes a rigid layer of adsorbed ions and a diffusion layer, in which ion diffusion may occur by thermal motion.

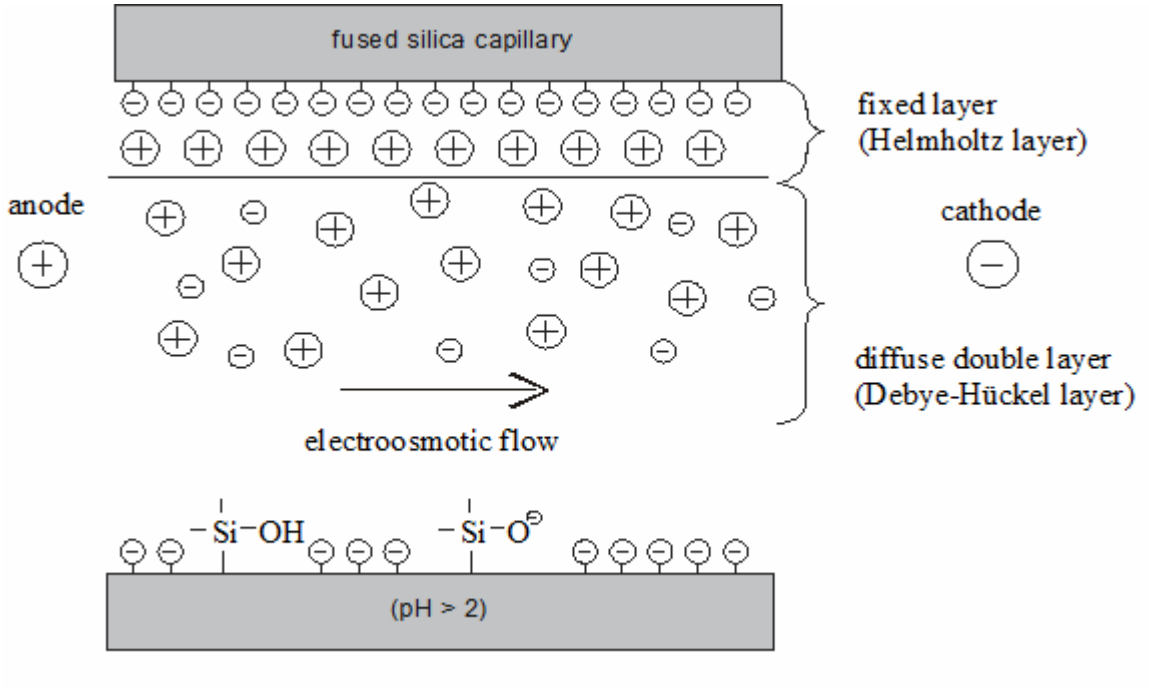


Fig. 6. Stern's model for generating a zeta potential and EOF [6]

When a voltage is applied across the capillary, cations on the diffusion layer migrate towards the cathode. This movement, which carries the whole bulk solution with an equal velocity, is described by comparation of the electric field strength with plug profile. It can be expressed by

$$\upsilon_{eof} = \left(\frac{\varepsilon \cdot \varsigma}{4 \cdot \pi \cdot \eta} \right) \cdot E \qquad \text{(Eq. 13)}$$

ε = the dielectric constant

ς = the zeta potential

η = viscosity of the buffer [Pa.s]

E = applied electric field [V.m^{-1}]

According to Eq. 13, the dielectric constant, viscosity of the buffer and the size of the zeta potential are the main factors that influence the mobility of EOF. For examples, solution viscosity depends on the temperature and leads to the difference in the EOF mobility. Therefore, temperature on the capillary is important to be controlled. The use of buffer additives and/or other modifications may effect the dielectric constant and viscosity of the buffer, depending on the nature of additive.

Since the zeta potential is proportional to the charge density on the capillary wall, the mobility of EOF is highly dependent on the electrolyte pH. Below pH 2, the ionization of silanols is small, and the EOF mobility is therefore not significant. Above pH 9, the silanols are completely ionized and the EOF mobility is strong. The pH dependence of the EOF mobility using fused-silica capillary is shown in Figure 7.

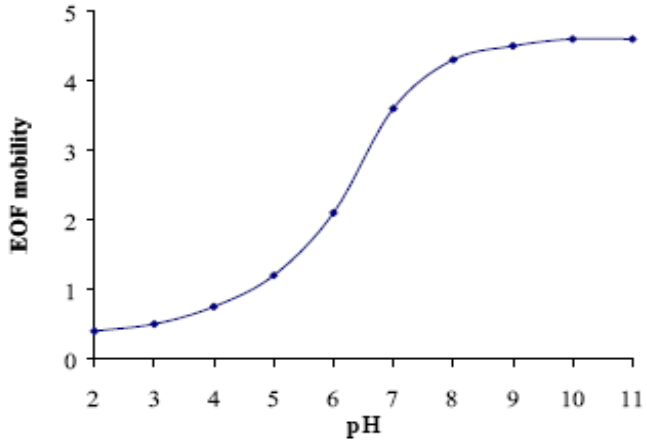


Fig. 7. The variation of EOF mobility with different pH [7]

EOF enables the simultaneous analysis of cations, anions, and neutral species during separation. Since the EOF mobility is sufficiently strong at pH > 7, their electrophoretic mobilities are smaller than electroosmotic mobility ($\mu_{ep} < \mu_{eo}$). Under this condition, most of the molecules will migrate in the same direction towards the cathode. Therefore, most of the sample molecules are be detected in the detector window.

When the voltage is applied, EOF moves from the anode to the cathode. At the same time, neutral compounds migrate with the same velocity as the EOF, cations migrate faster than the EOF and anions migrate more slowly than the EOF (Figure 8).

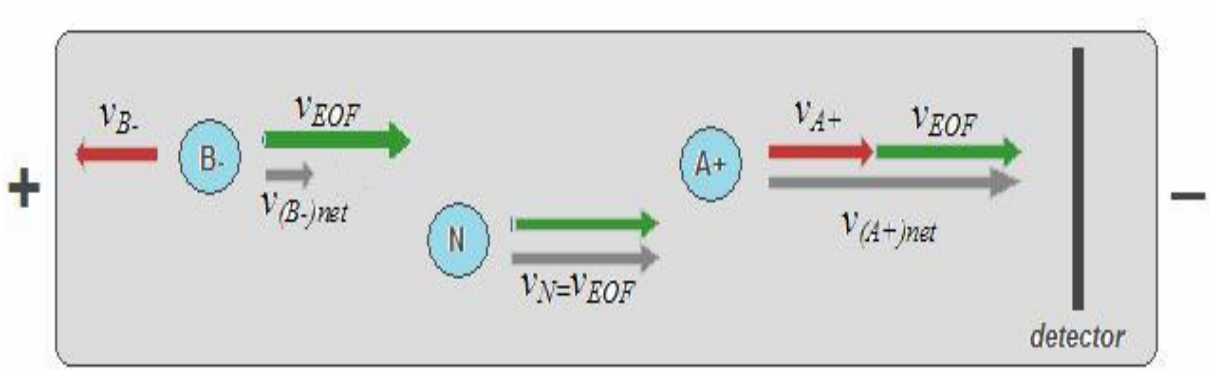


Fig. 8. Electroosmotic and electrophoretic mobility for most of molecules

As shown in Figure 8, the overall migration time is related to a combination of the electrophoretic mobility and the EOF mobility. Therefore, a solute's apparent electrophoretic mobility (μ_a) that is measured from the observed migration time is the sum of the effective electrophoretic mobility (μ_i) and the EOF mobility (μ_{eof}) (Eq. 14).

$$\mu_a = \mu_i + \mu_{eof} \qquad \text{(Eq. 14)}$$

1.2.3. Determination of effective mobility

The effective mobility of components that are separated in fused silica capillary by CZE can be calculated from an electropherogram. A sample that consists of a cationic component (B^+), an anionic component (A^-) and a neutral substance (N) will move in the presence of EOF. The neutral substance usually serves as an electroosmotic flow marker (EOF marker). In general, the net velocity $v_{i(net)}$ of component *i* is calculated by dividing the length of the capillary from the injection point to the detector by the migration time. The electrophoretic velocity v_i can be calculated from the net velocity and the electroosmotic flow velocity as follows:

$$v_i = v_{i(net)} - v_{eof} = \frac{L_D}{t_i} - \frac{L_D}{t_{eo}} \qquad \text{(Eq. 15)}$$

L_D = capillary length to detector or effective capillary length [cm]

t_i = migration time of component *i* [s]

t_{eof} = migration time of EOF marker [s]

$v_{i(net)}$ = net velocity of component *i* [cm.s^{-1}]

v_{eof} = electroosmotic flow velocity [cm.s^{-1}]

The effective electropherotic mobility of component *i* is then given by:

$$\mu_i = \frac{v_i}{E} = \frac{v_i \cdot L_T}{U} \qquad \text{(Eq. 16)}$$

L_T = distance between the electrodes or total capillary length [cm]

U = applied voltage [V]

If a CE is performed in the absence of the electroosmotic flow, where v_i is equal to $v_{i(net)}$, the following simplified equation can be used instead of Eqs. 15 and 16 [5]

$$\mu_i = \frac{v_i}{E} = \frac{L_D \cdot L_T}{t_i \cdot U} \qquad \text{(Eq. 17)}$$

1.2.4. Flow profile in CE

The charge on the capillary wall causes the driving force of EOF that is distributed uniformly along the length of the capillary. Consequently, no pressure drop and flow velocity is obtained (shown in Figure 9). This profile minimizes zone broadening and increases the efficiency. On the contrary, when a pressure is used as like for applying the external pump of HPLC, frictional forces at the column wall result in pressure drop along the capillary. It yields a parabolic or laminar flow profile in which a flow velocity occurs with the quickest parts in the middle of the capillary and approaching zero at the capillary wall (Figure 9).

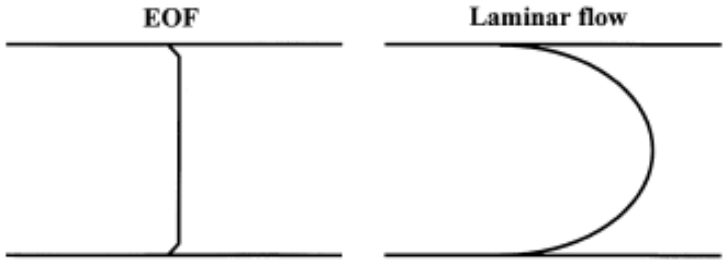


Fig. 9. Flow profile of EOF and laminar flow [7]

1.3. Capillary isoelectric focusing

1.3.1. General aspects of CIEF

Capillary isoelectric focusing is a high-resolution technique to separate amphoteric biomolecules. In principle, CIEF is used to analyze samples based on their isoelectric points at a pH gradient formed by carrier ampholytes (amphoteric electrolytes) under the influence of an electric field. Until recent days, CIEF methods did not provide satisfactory precision and reliability to enable its use for routine biomolecule analysis. In order to make this technique acceptable for routine analysis, to improve its reproducibility is still a major issue. Sample preparation, capillary selection, and focusing and mobilizing procedures are always developed, thus CIEF is able to become a more robust analytical method for many applications in biotechnology.

1.3.1.1. Application of CIEF

CIEF has been successfully applied in protein and peptide analysis including characterization, purification monitoring, evaluating stability, quantitative analysis and determination of the

isoelectric points of proteins [23-26]. CIEF is also routinely used in quality control laboratories to verify the identity and to ensure the stability of a protein.

New applications are frequently reported, in particular the use of CIEF in the analysis of biopharmaceutical products. In the biotechnology and biopharmaceutical industries, CIEF provides quantitative information on protein contents [24].
Many researches have reported on the application of CIEF, for analysis of erythropoietin glycoforms [27], analysis of the recombinant humanized monoclonal antibody HER2 (rhuMAbHER2) [28], evaluation of the separation in native haemoglobin [29]. It has been used also for estimation of the isoelectric points of proteins, such as human plasma proteins [25] and erythropoietin glycoforms [30].

CIEF has also the possibility of using a mass spectrometric (MS) detector for quantitative analysis of peptides and proteins. Kuroda used CIEF-MS to determine concentrations of peptides and proteins using angiotensin II and human-transferrin [31]. Storms also used CIEF-MS for the analysis of periplasmic proteins from Escherichia coli [32]. It showed a good accuracy and acceptable repeatability.

Nowadays, CIEF with whole-column imaging detection (WCID) is able to provide high resolution, high speed, and easy method development. It can be used for controlling the quality of products, monitoring structural changes (deamination, glycosylation, etc) during manufacturing process and storage. Liu has used this method to investigate the behaviour of MS2 virus and related antibodies [33]. Janini also has used an imaging CIEF assay for the determination of the identity, stability, and isoform distribution of a murine monoclonal antibody (MU-B3) [26].

1.3.1.2. Advantages of CIEF

The CIEF technique provides many advantages beside excellent resolution for protein separation, *i.e.* separations are carried out in a capillary format with an on-column detection and an automated analysis. In the recent days, imaging CIEF or CIEF with whole-column imaging detection (WCID) was developed to overcome the most frequent problems in CIEF. The mobilization step can distort a pH gradient established in the column by the focusing step. It results in poor reproducibility, longer analysis time, and degradation of the resolution of the focused zones. By using imaging CIEF, the entire capillary is imaged by a charge-

coupled device camera while the proteins are being focused in the capillary. The result provides high resolution, good reproducibility and reliability, and the mobilization step is not needed in this technique. The other advantages of imaging CIEF are the optimization of separation conditions is simplified and the separation time is greatly reduced [26, 33].

Viruses are mostly investigated by CZE. Nevertheless, CIEF has two significant advantages over than CZE. First, electric fields can affect the electrophoretic mobility of viruses in CZE, whereas the identification of viruses in CIEF is based only on p*I*, which is not influenced by an electric field. Second, the treatment of viruses in CIEF is potentially advantageous for safety reasons. An empty viral capsid is usually used as a model for studies of a virus to avoid the hazard of an infectious virus. It can be obtained by removing the genetic material from the virus. Based on the difference in charge and size, the electrophoretic mobility of the empty viral capsid in CZE is different from that of the virus. On the other hand, the p*I* value will remain the same for both and thus the separation can be performed by CIEF [33].

According to the principle on focusing, CIEF zones are extremely narrow and the technique typically provides higher resolving power than other CE separations [24]. Therefore, CIEF becomes the method of choice for the analysis of molecules with very close p*I* values [27]. Samples with a low concentration of amphoteric biomolecules are also suitable to be analyzed by CIEF [31].

1.3.1.3. Principle of CIEF

CIEF combines a high resolving power of conventional gel IEF with the automation and quantitation advantages of a CE instrument. Capillary isoelectric focusing is limited to the separation of amphoteric substances, because this technique uses different isoelectric points to separate the sample components.

The mechanism of isoelectric focusing is based on the formation of a stable pH gradient in the entire length of the capillary. The pH gradient is generated by the chemical compound of carrier ampholytes which have isoelectric points ranging from acidic to basic conditions in close proximity to each other under the application of high voltage.

The basic features of the instrument for CIEF are generally similar to CZE. The point of difference is that the anode compartment contains an acidic solution such as phosphoric or

aspartic acid (0.05 M), whereas the cathode compartment contains a base such as NaOH (0.02 M) or arginine (0.05 M).

A mixture of polyamino polycarboxylic acids is commonly used as ampholyte solution. When a voltage is applied, the mixture of ampholytes is separated in the capillary. Positively charged ampholytes migrate towards the cathode, while the negatively charged ones migrate towards the anode. In this condition, the pH decreases at the anodic section and increases at the cathodic section. When each ampholyte reaches its isoelectric point, the ampholyte migration ceases. Therefore, the great numbers of ampholytes in the solution produce a smooth pH gradient (Fig. 10).

After a protein sample is introduced into the capillary, voltage is applied and the protein will migrate along the pH gradient towards the position where its net charge is zero or the pH is equal to its p*I*. At this position, its velocity becomes zero, and the component will be focused into a narrow zone. The completion of the focusing process is indicated by a minimal current flow which does not change anymore (Fig. 11). The formation of the pH gradient and the focusing of the analytes can be established simultaneously, because both separation mechanisms are the same. In principle, the CIEF separation is represented in Fig. 12 [5, 24].

After the analyte focusing has been completed, the entire gradients have to be moved through the detector cell for the detection of the analytes bands. Several procedures of mobilization are discussed in 1.3.3.

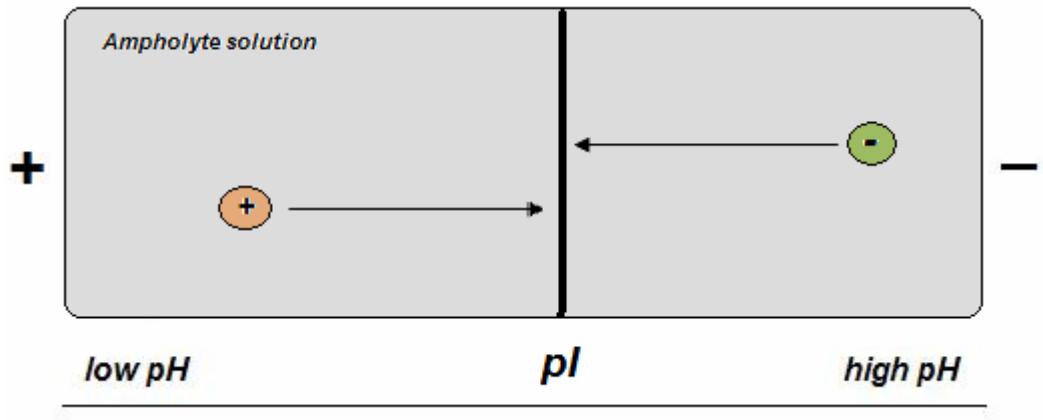


Fig. 10. Principle of isoelectric focusing

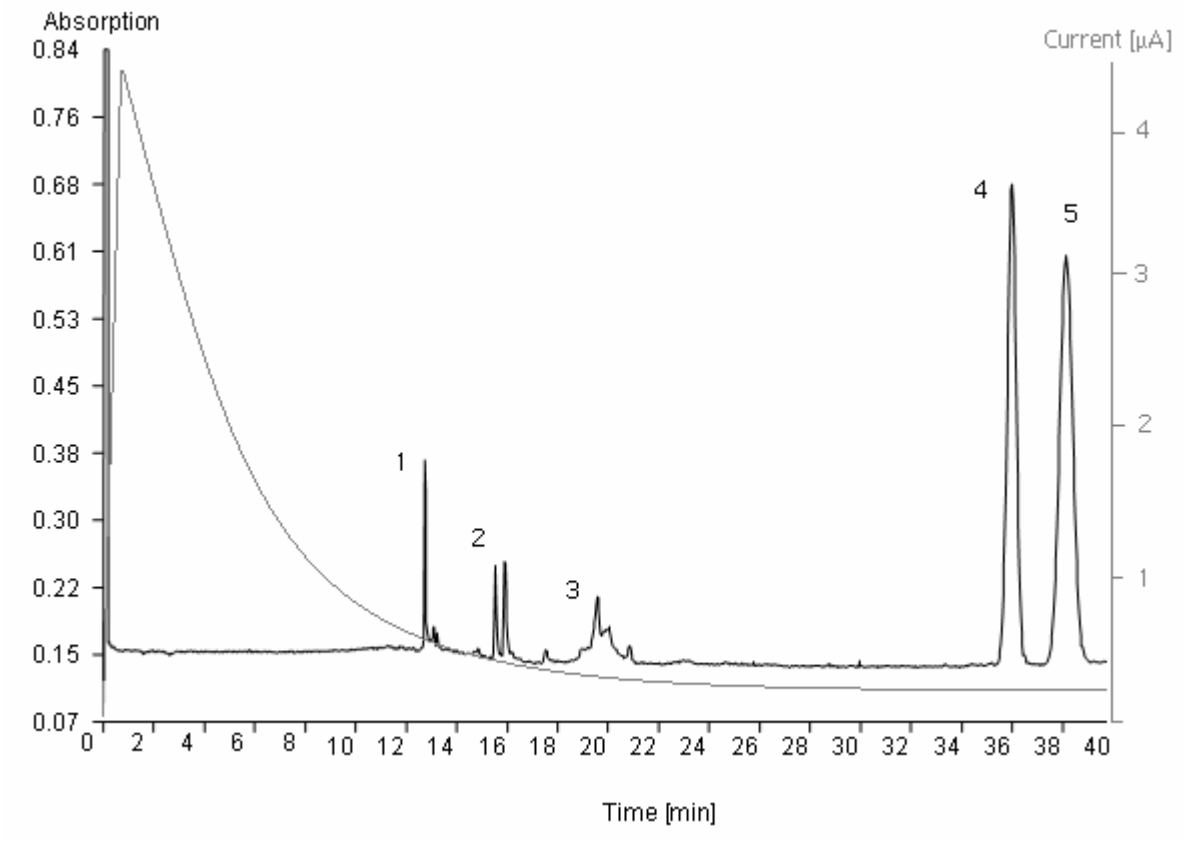


Fig. 11. Monitoring current during single-step CIEF

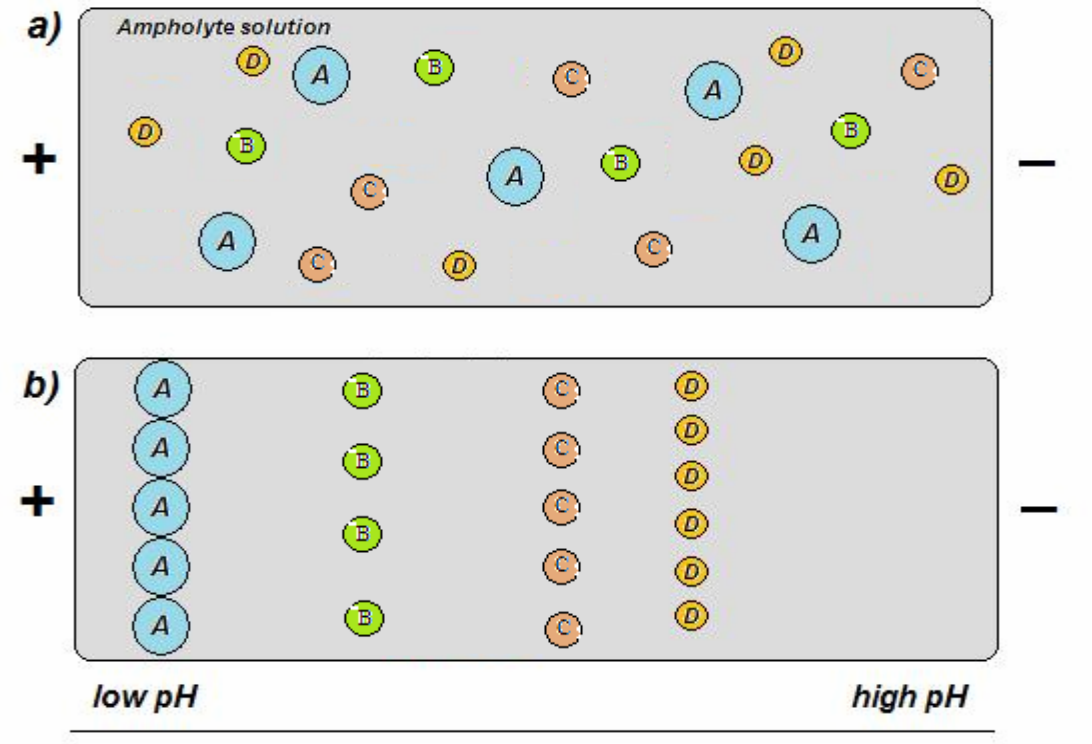


Fig. 12. Sample separations in CIEF

1.3.2. Detection

Most CIEF applications employ on-line detection of ultraviolet or visible absorbance for protein detection. The limitation of using UV absorbance for protein separations is that the ampholyte can absorb strongly at the wavelength below than 240 nm, as shown in Fig. 13. Nevertheless, the low-UV absorbance can provide information about the ampholyte distribution in the capillary. In order to avoid the detection of ampholytes in CIEF, UV absorbance at 280 nm is generally used to monitor protein separations. Visible absorbance is also possible to detect proteins especially for proteins that have chromophores, such as haemoglobin and cytochromes. By using imaging CIEF, the entire capillary can be used for the detection while the proteins are being focused in the capillary [2].

The application of UV detection limits both accuracy and sensitivity of the quantification, especially for analytes with a lack of unique chromophores. In order to overcome this problem, a MS detector is therefore applied. A good accuracy and acceptable repeatability are obtained using CIEF-MS for a complex protein mixture and quantitative analysis of peptides and proteins [31, 32].

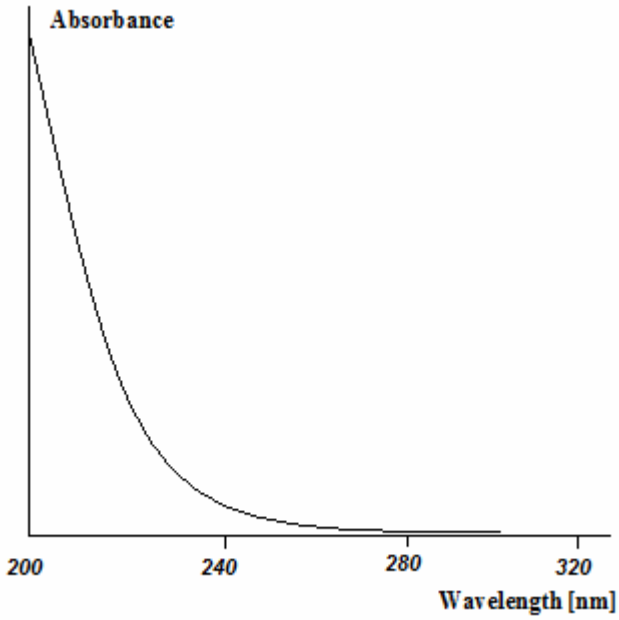


Fig. 13. UV absorption of ampholytes (Biolyte 3-10) [2]

1.3.3. Mobilization

In single-step CIEF, proteins are focused while they are transported towards the detector by EOF. The uncoated capillaries are usually used to produce the EOF. Nevertheless, these capillaries have disadvantages: the EOF is too high and strongly pH-dependent. The resolution of proteins separation and the decreasing linearity curved p*I* values against the migration time are observed in this single-step CIEF. The deviation from linearity can be improved by reducing EOF, for example using dynamic deactivation of the capillary surface with addition of a surfactant.

In order to avoid deviations of the p*I* curve, a constant mobilization velocity is desirable. It can be achieved by applying two-steps CIEF. In an initial step, a focusing is performed, and after attainment of equilibrium, the focused zones are mobilized towards the detection window in a second step [24]. There are two techniques that have been described to mobilize focused zones.

1.3.3.1. Chemical mobilization

In this technique, the mobilization is achieved by changing the composition of anolytes or catholytes. This causes a shift of the pH gradient and then the focused zones migrate electrophoretically through the detection window.

For anodic mobilization, the hydronium ions are replaced by sodium ions in the anolyte, whereas the hydroxyl ions are replaced by chloride ions in the catholyte for cathodic mobilization. When high voltage is applied, the addition of ions alters the pH in the capillary. The change of the pH leads both ampholytes and analytes to be mobilized in the direction of the reservoir with added ions. The choice of the mobilization technique and reagents depends on p*I* of the analytes and the mobilization time depends on the concentration of ions in the respective electrolytes. Zwitterions are usually used to produce a more effective mobilization of the protein zones across a wide pH gradient in where effective zwitterions depend on selection of the mobilization reagent [24, 34].

The principle of chemical mobilization is described in following equations below.

$$C_{H3O}^{+} + \Sigma \quad C_{NH3}^{+} = C_{OH}^{-} + \Sigma \; C_{COO}^{-} \qquad \text{(Eq. 18)}$$

$$C_{X}^{n+} + C_{H3O}^{+} + \Sigma \; C_{NH3}^{+} = C_{OH}^{-} + \Sigma \; C_{COO}^{-} \qquad \text{(Eq. 19)}$$

$$C_{H3O}^{+} + \Sigma \; C_{NH3}^{+} = C_{OH}^{-} + \Sigma \; C_{COO}^{-} + Y^{m-} \qquad \text{(Eq. 20)}$$

C_{H3O}^{+} = concentrations of hydronium ions

C_{OH}^{-} = concentrations of hydroxyl ions

C_{NH3}^{+} = concentrations of positive groups in the ampholytes

C_{COO}^{-} = concentrations of negative groups in the ampholytes.

Eq. 18 expresses the electroneutrality condition at steady state in the capillary during focusing. In this condition, the concentration of hydronium ions and positive groups in the ampholyte is equal to the concentration of hydroxyl ions and negative groups. In anodic mobilization, the addition of a non-proton cation X^{n+} to the anolyte reservoir (left side of the equation) will result migration of the non-proton cation X^{n+} into the capillary. As a consequence, a reduction of the hydronium ions concentration or an increased pH in the capillary takes place as written in Eq. 19. On contrary in cathodic mobilization, the addition of a non-hydroxyl ion Y^{m-} to the catholyte yields the migration of the non-hydroxyl ion Y^{m-} into the capillary. Then, a reduction of the hydroxyl concentration or a decreased pH in the capillary occurs as written in Eq. 20 [35]. This alternation of the pH-gradient (anodic or cathodic mobilization) will result in the mobilization of ampholytes and analytes passing through detector window as shown at Fig. 14 [2].

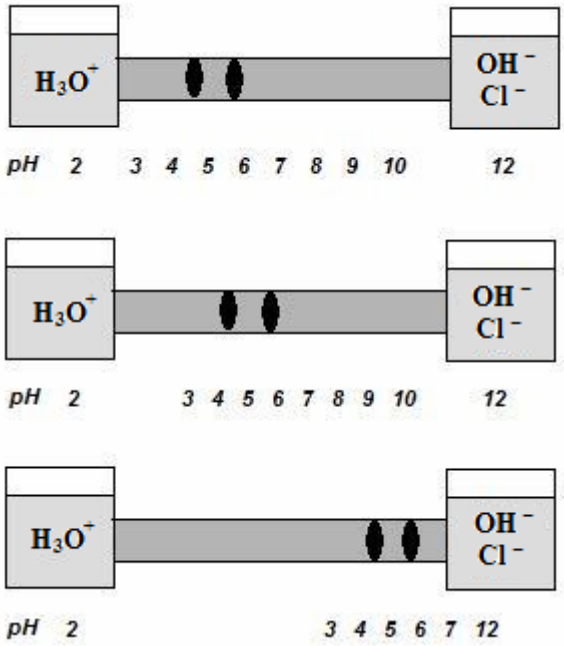


Fig. 14. Cathodic mobilization

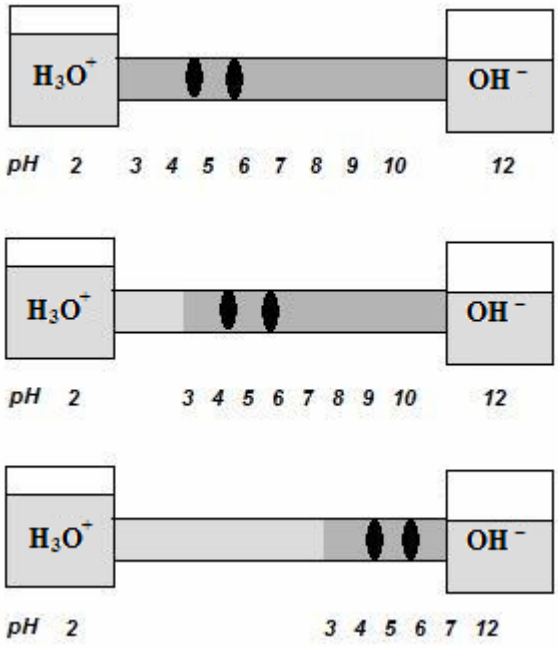


Fig. 15. Hydraulic mobilization

1.3.3.2. Hydraulic mobilization

The second technique to transport focused zones towards the detection window is to apply pressure or vacuum or gravity at the one end of the capillary after the proteins have been focused in a first step.

The pressure mobilization using an HPLC pump is usually used to accomplish the displacing of the focused zones in the capillary. Another mobilization can either be created by the height difference of the liquid contained in the reservoir (gravity mobilization) or by applying vacuum at the capillary outlet (vacuum mobilization). The principle of hydraulic mobilization is presented in Fig. 15. This hydraulic mobilization produces a parabolic flow profile which may reduce the resolution of the separation. In order to avoid a loss of resolution, it is necessary to apply an electric field across the capillary. Hence, focused protein zones can be maintained during mobilization [24, 34].

In general, chemical mobilization produces sharper peaks in the neutral to basic end of the gradient (protein zones focused closer to the detector), whereas hydraulic mobilization increases resolution at the far end of the capillary (acidic proteins). For proteins with neutral p*I* values, chemical mobilization preserves resolution better than hydraulic mobilization. Chemical mobilization offers the highest resolution and good linearity of the p*I* plotted against migration time curve comparable to hydraulic mobilization, especially for the separation of proteins with small p*I* differences [2].

1.3.4. Some crucial parameters

1.3.4.1. Ampholyte selection

In order to produce the desired resolution, a number of requirements related to the carrier ampholyte should be given, *i.e.* p*I* values of the amphoteric character in the pH range of interest, high conductivity to carry the current, low UV absorbance, no interactions with the analyte and high solubility in water.

A mixture of polyamino polycarboxylic acid is commonly used as ampholyte solution. Typical concentrations of ampholytes are 1 – 2%. For complex samples or proteins with wide isoelectric points, an ampholyte mixture in a wide range pH 3-10 is frequently used, whereas narrow-range mixtures are used to achieve high resolution of protein separation with limited

p*I* range. Nevertheless, a disadvantage is also found in these carrier ampholytes because it can disturb the detection of analytes. The carrier ampholytes have a high UV absorption at short wavelengths, therefore detection at 280 nm is necessary. At this wavelength, the ampholyte has a low absorbance [5].

1.3.4.2. Effect of salt

CIEF has several limitations in the presence of salt in the sample-ampholyte mixture. It changes the distribution of the pH gradient during focusing, increasing the time required for focusing and causing peak broadening during the mobilization. The high initial current which is due to the presence of salt can increase the risk of overheating and loss of resolution. During the focusing step, heat generation that is concentrated in focused zones may lead to protein precipitation. If biological samples contain salt, a desalting procedure prior to CIEF is recommended [24, 36].

Simple desalting procedures have been described, such as sample dilution with the ampholyte mixture if the protein is present in a high concentration and dialysis or ultrafiltration for low protein concentration. This procedure is effective to remove salt from biological samples, although additional cost in time and material is high [24].

1.3.4.3. Protein precipitation

Protein precipitation is a major problem in capillary isoelectric focusing. Proteins become highly concentrated at zero-net-charge conditions because of promoting aggregation and loss of solubility during the focusing process. Precipitation in CIEF is evidenced by current loss or fluctuation, poor reproducibility of peak height and migration time. Very sharp peaks or spikes are seen in electropherogram. In the worst case, precipitation can block the capillary and current falls to zero [37].

Protein precipitation can be minimized by reducing the focusing time or the protein concentration. The most effective way of reducing protein precipitation is the addition of protein solubilizing agents such as neutral or zwitterionic reagents (glycols, sulphobetaines, taurine, N,N-bis(2-hydroxyethyl)glycine (Bicine), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)). The addition of sugars or their derivates, such as saccharose, sorbitol and sorbose can improve the protein solubility on the proximity of the p*I* [38]. Nonionic

surfactants such as Triton X-100, Brij-35 and Tween can also minimize protein precipitation [37].

1.3.4.4. Internal standard

The use of CIEF as a routine analysis technique has been improved, mainly since the reproducibility has not showed as good as in other modes of CE, especially concerning migration times. One approach which shows great improvement of the reproducibility in CIEF is using internal standards as co-migrating analyte. The internal standard can be used to characterize the analyte by recognizing their p*I* values. The use of internal standards is also able to improve the precision of peak area and migration time determinations through reduction of errors in injection, diluting, sample pre-treatment or solvent evaporation [39].

Internal standard for CIEF should be ampholytes, highly soluble in water, sharply focusing, stable, highly pure, with known p*I* values, high absorption at the detection wavelength, and be non-reactive with sample compounds or the ampholyte [24].

Many substances such as amino acids and derivates, peptides and derivates, synthetic peptides, and native proteins were tested as internal standards for CIEF [23, 25, 40-42]. Native proteins with high molecular mass have some distinct disadvantages. They tend to precipitate at pH values close to their p*I*, produce multiple peaks and show instability in aqueous solutions. Because of these reasons, mainly reference substances with low molecular mass have been reported [41, 42]. Only a few of them show suitable UV absorption at wavelength 280 nm which is usually used for protein detection by isoelectric focusing [41].

1.4. Capillary electrophoresis of proteins

Based on three dimensional conformations, a protein is determined by the sequence of amino acid components and their interactions with each other. As a consequence, proteins differ from one another in shape and size. Heterogeneity of the protein surface is also found, *i.e.* it has possibly hydrophilic, hydrophobic, cationic and anionic patches at the same time. The distributions of these different patches on the protein surface are greatly affected by environmental factors such as pH, ionic strength, temperature and interaction with organic molecules or a solid surface. Because of these factors, electrostatic force, hydrogen bonding,

charge-transfer, and/or hydrophobic interactions possibly happen and it can cause protein adsorption.

For the analysis of proteins, CE is considerable as powerful technique because it is not a single technique which has different mechanisms of separation. Many techniques of CE and their combinations can be used for the analysis of proteins, such as CZE and reversed-charge CZE (RE-CZE), CIEF, MEKC, sodium dodecyl sulphate-gel CE (SDS-gel CE), ITP, and combinations with HPLC, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS), and electrospray ionization-mass spectrometry (ESI-MS). The choice of a CE technique depends on the aim of analysis.

Many publications discuss about CE techniques to improve the separation of proteins. As a result, it can be used for the analysis of proteins in real samples, such as biological tissue, protein pharmaceuticals, food and agricultural samples. Many topics such as the electrophoretic migration of proteins, sample pre-treatment, choosing the CE techniques and various forms of detection have been reported to be able to support an improvement of proteins separation [43].

1.4.1. Protein adsorption

The interaction between positively charged proteins and negatively charged silanol groups on the capillary surface is a major problem for the analysis of proteins by capillary electrophoresis, especially if the proteins are separated at pH values lower than their isoelectric points (p*I*). Consequently, adsorptions at the capillary wall frequently happen. This can cause peak broadening and asymmetric peak shapes, low efficiency, low recovery of analysis, irreversible protein adsorption, a drifting EOF and irreproducible migration times [39, 44].

1.4.2. Strategies for preventing protein adsorption

Several strategies have been proposed to prevent the problem of protein adsorption. In CZE, the choice pH buffer is able to influence the charges of analytes. pH condition close to and less than p*I* of proteins are able to increase amount of possible binding sites thus proteins have a stronger tendency to be adsorbed to the capillary wall. Therefore, extreme pHs or pH values higher than the protein p*I* are favorable to overcome the adsorption problem. The use of

extreme basic or acidic pHs give the same sign of the capillary wall and the proteins. They repel each other and the adsorption can be prevented.

The addition of high concentrations of alkali salts, zwitterions or other additives to the buffer solution can be used to suppress the electrostatic interaction between the capillary wall and the proteins. The high concentrations of positively charged ions compete positively charges of the protein to interact with the negative silanol groups of the capillary wall. Zwitterions perform ion pairing with the proteins, thus protein-wall and protein-protein interactions can be reduced. The use of buffer additives is also useful for masking the activity of silanol groups. Nevertheless, in the addition of ionic salt, the applied field strength should be controlled to avoid high current that may possibly lead to denaturation and precipitation of proteins [39, 44].

In order to deactivate the silanol groups, the use of coated capillaries in CZE is preferable to reduce the wall interactions of protein molecules. The ideal coating can provide separation efficiency, better protein recovery and reproduciblility of EOF and migration time of analytes. Yet, significant adsorption of proteins is still observed in recent days using coated capillaries [19, 45]. In order to improve the separation efficiency when using coated capillaries, many factors (temperature, ion strength, pH, composition of BGE, chemical and structural properties of the capillary surface, rinsing procedure, etc) should also be considered [43, 46].

On the other side, the stability of a protein is also one of the determinants related to the adsorption behaviour and can even be one of the driving forces for protein adsorption. Therefore, the possible way to reduce protein adsorption in this case is by increasing the stability of proteins [47]. In a recent study, sugar excipients such as trehalose, mannitol, sucrose and sorbitol have shown a decrease of protein adsorption by stabilizing the native state of the protein in the solution [48-50].

2. Experimental

2.1. Capillary zone electrophoresis

2.1.1. Chemicals

ß-lactoglobulin (bovine milk, p*I*: 4.83-5.4 [45, 51], M_r: 18.4 kDa), cytochrome *c* (horse heart, p*I*: 9.59 [45], M_r: 11.7 kDa), ß-casein (bovine milk, p*I*: 4.6 [45], M_r: 24 kDa), neostigmine bromide, and sucrose were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium acetate anhydrous, trehalose, sodium dodecyl sulfate (SDS), sodium chloride and acetanilide were purchased from Fluka (Steinheim, Germany); disodium hydrogen phosphate-2-hydrate and potassium dihydrogen phosphate from Riedel-de Haën (Sigma-Aldrich, Seelze, Germany). Poly(ethylene glycol) (PEG) 20000, hydrochloric acid, 2-propanol, acetic acid, phosphoric acid, sodium formate and formic acid were purchased from Merck (Darmstadt, Germany).

2.1.2. Solutions

ß-lactoglobulin, cytochrome c and ß-casein as model proteins were freshly prepared using isoosmotic NaCl 0.9% m/V solution. Acetanilide as an EOF marker and neostigmine bromide as an internal standard were dissolved in buffer solution. The sample solutions were prepared by mixing 0.8 mL of this protein solution and 3.2 mL of the acetanilide and neostigmine bromide. The total concentration of analytes in the sample solution was 100 µg/mL of acetanilide, 500 µg/mL of neostigmine bromide and 35 µmol/L of protein.

The 50 mmol/L acetate buffers with pH 5.5, 5.0, 4.5 and 4.0 were prepared by weighing the appropriate amounts of sodium acetate and acetic acid and filling up to volume; the 50 mmol/L phosphate buffer with pH 7.0, 6.5, 6.0 consisted of disodium hydrogen phosphate-2-hydrate and potassium dihydrogen phosphate; likewise, the 50 mmol/L formate buffers with pH 3.5 consisted of sodium formate and formic acid. Complete-buffer system is shown in the Table 1.

Trehalose with the concentration 35 and 70 µmol/L and sucrose with the concentration 35 µmol/L as protein stabilizer were prepared on the protein sample and running buffers. The rinsing solution with a content of NaOH 1 mol/L, 2-propanol 10% and SDS 200 mmol/L was prepared to regenerate the capillary after 1 series of protein separation. Additive buffer was

made by dilution of PEG into the buffer solution with the final concentration 3.2 and 32 mg/mL.

For the experiment of protein analysis using a LPA-coated capillary with HCl rinsing, ß-lactoglobulin as protein sample with a high concentration (175 µmol/L) and HCl (2 and 3 mol/L) as well as phosphoric acid (85% (m/m)) as rinsing reagents were used.

All solutions used were prepared with doubly-distilled water and were filtered through Rotilabo®-syringes filters with using a pore size of 0.22 µm (Carl Roth, Karlsruhe, Germany) to prevent capillary blockage. Except for protein solutions, all other solutions were degassed in an ultrasonic bath.

Table 1.Buffer system

Buffer System	pH	Concentration (mmol/L)	Procedures
Phosphate	7.0	50	420.69 mg KH_2PO_4 + 339.25 mg $Na_2HPO_4.2H_2O$ filled up to 100 mL
Phosphate	6.5	50	568.91 mg KH_2PO_4 + 145.22 mg $Na_2HPO_4.2H_2O$ filled up to 100 mL
Phosphate	6.0	50	640,76 mg KH_2PO_4 + 51.17 mg $Na_2HPO_4.2H_2O$ filled up to 100 mL
Acetate	5.5	50	341.80 mg CH_3COONa + 47.26 µL CH_3COOH filled up to 100 mL
Acetate	5.0	50	251.39 mg CH_3COONa + 110 µL CH_3COOH filled up to 100 mL
Acetate	4.5	50	136.89 mg CH_3COONa + 189 µL CH_3COOH filled up to 100 mL
Acetate	4.0	50	56.10 mg CH_3COONa + 240 µL CH_3COOH filled up to 100 mL
Formate	3.5	50	122.40 mg HCOONa + 136 µL HCOOH filled up to 100 mL

2.1.3. Instrumentations

The instrumentation for protein analysis using CZE technique was UniCAM Crystal 310 CE (UniCAM Ltd., Cambridge, UK), equipped with a UV detector (wavelength 210 nm). Bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) had dimensions of 60 cm total length, 48 cm effective length, and 50 µm inner diameter (i.d.). The linear polyacrylamide (LPA)-coated capillaries were from Polymicro Technologies, Phoenix, AZ, having a total length of 60 cm, an effective length of 48 cm, and i.d. 75 µm. On the other hand, PDMAA-coated capillaries made by dimethylacrylamide (DMAA) polymerization with

an internal diameter of 75 µm (IMTEK, Albert-Ludwigs University of Freiburg, Germany) were cut to an effective length of 48 cm from the total length of 60 cm. During all experiments, the thermostat was set to 25°C. All integration was done by an integration program C.I.S.S. (Correct Integration Software System), (Würzburg, Germany) [52].

2.1.4. Methods

2.1.4.1. Analysis of ß-lactoglobulin using PDMAA-coated capillary

PDMAA-coated capillaries were rinsed by buffer with the pressure 1200 mbar for 30 minutes. The separation method was started by rinsing with buffer for 2 minutes and then was followed by hydrodynamic injection of the sample by applying a pressure of 30 mbar for 12 seconds. The protein separation was performed by applying a voltage of 13 kV and additional pressure of 40 mbar (Table 2).

Table 2. Separation method of proteins using PDMAA-coated capillary

pH		Number of runs	t_{inj} [s]	P_{inj} [mbar]	V [kV]	I [µA]	P_{add} [mbar]
7.0	Control 1	30	12	30	13	~97	40
	Protein	30	12	30	13	~97	40
	Control 2	30	12	30	13	~97	40
6.5	Control 1	30	12	30	13	~106	40
	Protein	30	12	30	13	~106	40
	Control 2	30	12	30	13	~106	40
6.0	Control 1	30	12	30	13	~92	40
	Protein	30	12	30	13	~92	40
	Control 2	30	12	30	13	~92	40
5.5	Control 1	30	12	30	13	~19	40
	Protein	30	12	30	13	~19	40
	Control 2	30	12	30	13	~19	40

Control 1 and Control 2: the analysis of sample content of internal standard and EOF marker.
Protein: the analysis of sample content of protein, internal standard and EOF marker.

2.1.4.2. Analysis of ß-lactoglobulin using bare fused-silica capillaries without and with the presence of trehalose or sucrose

The bare fused-silica capillaries were rinsed with 1 mol/L NaOH for 2 hours, buffer for 30 minutes with the pressure of 1200 mbar, and continued by equilibration of the buffer for

2 hours with the voltage of 25 kV. The separation method was started by rinsing with buffer for 2 minutes and was then followed by hydrodynamic injection of the sample by applying a pressure of 30 mbar for 12 seconds. The protein separation was performed by applying a voltage of 25 kV (Table 3). In some experiments, a rinsing solution consisting of NaOH 1 mol/L, 2-propanol 10% and SDS 200 mmol/L was used to regenerate the capillary after 1 series of protein separation.

Table 3. Separation method of proteins without and with the presence of trehalose or sucrose

pH		Number of runs	t_{inj} [s]	P_{inj} [mbar]	V [kV]	I [µA]
6.5	Control 1	30	12	30	25	~62
	Protein	30	12	30	25	~62
	Control 2	30	12	30	25	~62
6.0	Control 1	30	12	30	25	~56
	Protein	30	12	30	25	~56
	Control 2	30	12	30	25	~56
5.5	Control 1	30	12	30	25	~34
	Protein	30	12	30	25	~34
	Control 2	30	12	30	25	~34

Table 4. Separation method of proteins with the presence of PEG

Concentration of PEG	pH		Number of runs	t_{inj} [s]	P_{inj} [mbar]	V [kV]	I [µA]	P_{add} [mbar]
-	6.0	Control 1	30	12	30	25	~56	-
		Protein	30	12	30	25	~56	-
		Control 2	30	12	30	25	~56	-
3.2 mg/mL	6.0	Control 1	30	12	30	25	~68	-
		Protein	30	12	30	25	~68	-
		Control 2	30	12	30	25	~68	-
32 mg/mL	6.0	Control 1	30	12	30	18	~75	100
		Protein	30	12	30	18	~75	100
		Control 2	30	12	30	18	~75	100
	5.0	Control 1	30	12	30	18	~72	100
		Protein	30	12	30	18	~72	100
		Control 2	30	12	30	18	~72	100
	4.0	Control 1	30	12	30	18	~46	100
		Protein	30	12	30	18	~46	100
		Control 2	30	12	30	18	~46	100

Table 5. Separation method of proteins using LPA-coated capillary with HCl and phosphoric acid as rinsing agents

pH		Concen-tration	Rinsing reagent	Number of runs	t_{inj} [s]	P_{inj} [mbar]	V [kV]	I [µA]	P_{add} [mbar]
5.5	Control 1	-	-	30	12	30	20	~60	15
	ß-lactoglobulin	35 µM	2 M HCl	30	12	30	20	~60	15
	Control 2	-	-	30	12	30	20	~60	15

Protein	Concen-tration	pH	Rinsing reagent	Number of runs	t_{inj} [s]	P_{inj} [mbar]	V [kV]	I [µA]	P_{add} [mbar]
ß-lactoglobulin	35 µM	5.5	2 M HCl	60	12	30	20	~60	15
ß-lactoglobulin	175 µM	5.5	2 M HCl	60	12	30	20	~60	15
ß-lactoglobulin	175 µM	5.5	3 M HCl	60	12	30	20	~60	15
ß-lactoglobulin	175 µM	5.5	85 % (m/m) H_3PO_4	60	12	30	20	~60	15
ß-casein	35 µM	5.5	2 M HCl	60	12	30	20	~60	15
cytochrome c	35 µM	5.5	2 M HCl	60	12	30	20	~60	15
ß-lactoglobulin + cytochrome c	each 35 µM	5.5	2 M HCl	60	12	30	20	~60	15
ß-casein	35 µM	4.5	2 M HCl	30	12	30	30	~43	15
cytochrome c	35 µM	4.5	2 M HCl	30	12	30	30	~43	15
ß-casein	35 µM	3.5	2 M HCl	30	12	30	20	~38	25
ß-casein	35 µM	3.5	85 % (m/m) H_3PO_4	30	12	30	20	~38	25
cytochrome c	35 µM	3.5	2 M HCl	30	12	30	30	~54	10

Protein	Concen tration	pH	Rinsing reagent	Number of runs	t_{inj} [s]	P_{inj} [mbar]	V [kV]	I [µA]	P_{add} [mbar]
ß-lactoglobulin	35 µM	5.5	2 M HCl	230	12	30	20	~60	15
cytochrome c	35 µM	4.5	2 M HCl	226	12	30	30	~43	15
ß-casein	35 µM	3.5	2 M HCl	135	12	30	20	~38	25
ß-casein	35 µM	3.5	85 % (m/m) H_3PO_4	120	12	30	20	~38	25

2.1.4.3. Analysis of ß-lactoglobulin, cytochrome c and ß-casein using bare fused-silica capillaries with the presence of PEG as a buffer additive

New bare fused-silica capillaries were previously conditioned with 1 mol/L NaOH for 2 hours, continued by a rinsing with buffer for 30 minutes (1200 mbar), then equilibrated for 2 hours with the applied voltage, and afterwards directly used for protein analysis. The analysis was started with buffer rinsing at 1200 mbar for 2 minutes. Next, samples were hydrodynamically injected by applying a pressure of 30 mbar for 12 seconds (Table 4). In

some experiment, after each series, the capillary was reconditioned like a new capillary to regenerate it.

2.1.4.4. Analysis of ß-lactoglobulin, cytochrome c and ß-casein using LPA-coated capillaries with HCl and phosphoric acid as rinsing agents

New linear polyacrylamide-coated capillaries were preconditioned with a buffer for 30 minutes and afterwards directly used for protein analysis. The analysis was started with buffer rinsing at 1000 mbar for 2 minutes. Next, the samples were hydrodynamically injected by applying a pressure of 30 mbar for 12 seconds. After each run, the capillary was rinsed for 4 minutes with buffer. After every 10^{th} run, it was also rinsed for 5 minutes with a strong acid (either 2 mol/L or 3 mol/L hydrochloric acid, or 85% (m/m) phosphoric acid), followed by a rinse with water for 5 minutes and then with buffer for 30 minutes. All rinses were done at 1200 mbar (Table 5).

2.2. Capillary isoelectric focusing

2.2.1. Chemicals

ß-lactoglobulin (bovine milk, p*I*: 4.83-5.4 [45, 51], M_r: 18.4 kDa), myoglobin (horse heart, p*I*: 6.8-7.4 [45, 51], M_r: 17.8 kDa), ovalbumin (chicken white egg, p*I*: 5.1 [45, 51], M_r: 43 kDa), hydroxypropylmethylcellulose (HPMC), L-tryptophane and 4-aminobenzoic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid, sodium chloride and phosphoric acid were purchased from Merck (Darmstadt, Germany). Pharmalyte™ 3-10 for isoelectric focusing was purchased from GE Healthcare Bio-Sciences AB (0.36 meq/mL pH, Uppsala, Sweden). L-tryptophan and 4-aminobenzoic acid (Sigma-Aldrich, Steinheim, Germany) were studied as possible internal standards.

2.2.2. Solutions

10 mmol/L phosphoric acid was used as anolyte and 20 mmol/L sodium hydroxide as catholyte. Isoosmotic sodium chloride 0.9% m/V was used to remove proteins from the capillary wall. The carrier ampholyte solution and the sample solution contained 0.8% m/V HPMC and 2% V/V Pharmalyte, equivalent to 7.2 µeq/mL carrier ampholyte. The proteins or/and internal standards were dissolved in bidistilled water and mixed with the carrier

ampholyte stock solution to the final concentration of myoglobin 0.3 mg/mL, ß-lactoglobulin 0.6 mg/mL ovalbumin 1.2 mg/mL, L-tryptophan 0.1 mg/mL and 4-aminobenzoic acid 0.02 mg/mL. HCl 2, 3, 6 mol/L and phosphoric acid 85% (m/m) were prepared as rinsing reagents. All solutions were prepared with doubly-distilled water, were filtered through Rotilabo®-syringes with a pore size of 0.22 µm (Carl Roth, Karlsruhe, Germany) to prevent capillary blockage, and then were degassed in an ultrasonic bath.

2.2.3. Instrumentations

The instrumentations employed were UniCAM Crystal 310 CE using a Spectra 100 UV detector (UNICAM Ltd., Cambridge, UK) and PrinCE 550 CE (Prince Technologies, Emmen, Netherland) with a Lambda 1010 UV detector (Bischoff, Leonberg, Germany). During the protein analysis by isoelectric focusing, a switch from UniCAM to PrinCE Instrument was performed. In principles, both instruments are similar. Nevertheless, the PrinCE Instrument provides a double lift system to permit the outlet vial automatically changes. The capillary length in the PrinCE Instrument is shorter and thus provides faster analysis time. Linear polyacrylamide-coated capillaries used in the UniCAM Instrument were from Polymicro Technologies, Phoenix, AZ, having a total length (L_{tot}) of 60 cm, an effective length (L_{det}) of 48 cm, and an internal diameter (i.d.) of 75 µm. The same capillaries with i.d. = 75 µm, L_{tot} = 85 cm, L_{det} = 31 cm were employed for the PrinCE Instrument. All separations were carried out at 20°C, 23°C or 25°C with detection at 280 nm. The program K.I.S.S was used to integrate the electropherograms [52].

2.2.4. Methods

2.2.4.1. Cleaning LPA-capillary surface

The capillary was first rinsed with carrier ampholyte solution for 4 minutes under 1030 mbar. The samples were then hydrodynamically injected by applying a pressure of 1030 mbar for 10 seconds. Using the UniCAM instrument, the focusing step was performed at a voltage of 30 kV (0.9 – 8.4 µA) for 10 minutes and continued with a mobilization step using a pressure of 30 mbar. After each run, the capillary was rinsed for 5 minutes and 2000 mbar pressure with sodium chloride 0.9% m/V. In order to remove adsorbed protein from the capillary wall, the capillary was also rinsed with 2 mol/L, 3 mol/L, 6 mol/L hydrochloric acid and

phosphoric acid 85% m/m for 5 minutes under 1030 mbar pressure, followed by rinses with water for 20 minutes under 1030 mbar pressure. The complete procedure of CIEF is shown in Table 6. At the end of the analysis day, the capillary was rinsed with water and then both capillary ends kept immersed into water vials.

Table 6. CIEF procedure using UniCAM instrument

Step	Prodecure	Pressure [mbar]	Voltage [kV]	Duration [min]
1	Filling carrier ampholyte	1030	-	4
2	Sample injection	1030	-	0.16
3	Focusing	-	30	10
4	Mobilization	30	30	unlimited
5	Rinsing with NaCl 0.9% m/V	2000	-	5
6	Rinsing with rinsing reagent	1030	-	5
7	Rinsing with water	1030	-	20
8	Conditioning with carrier ampholyte	1030	-	5

2.2.4.2. Investigation of further error sources

2.2.4.2.1. The fluctuation of room temperature

The procedure is similar to the Table 6. Only 3M hydrochloric acid as a preferable rinsing reagent was used for the sixth step. The influence of room-temperature fluctuation on the irreproducibility of migration time and peak area during protein separation with CIEF was investigated. The temperature of UniCAM instrument was programmed for this purpose on two different temperatures with 20°C or 25°C. The room temperature for each run of the protein analysis was observed.

2.2.4.2.2. Alteration of surface structure

2.2.4.2.2.1. Capillary maintenance

The capillary maintenance was investigated with the procedure given in Table 6. Only 3M hydrochloric acid as a preferable rinsing reagent was used for the sixth step. The capillary maintenance was performed to evaluate a change of capillary surface structures for the period of capillary storage in which it could affect day-to-day irreproducibility of migration time and peak area. In this experiment, water rinsing was performed by applying pressure (300 or 700 mbar) and voltage (30 kV) for period of capillary storage.

2.2.4.2.2.2. Consecutive runs

In order to avoid the alteration of capillary surface during capillary storage, the analysis runs were performed consecutively without interruption. A switch to another instrument (PrinCE 550 CE System) was performed to facilitate this consecutive runs. The focusing and mobilization step using the PrinCE Instrument was performed at a voltage of 30 kV (0.3 – 4.5 μA) without and with additional pressure (25 mbar). The complete procedure is shown in Table 7.

Table 7. CIEF procedure using PrinCE instrument

Step	Prodecure	Pressure [mbar]	Voltage [kV]	Duration [min]
1	Filling carrier ampholyte	1030	-	4
2	Sample injection	1030	-	0.16
3	Focusing & mobilization	-/25	30	unlimited
4	Rinsing with NaCl 0.9%	2000	-	5
5	Rinsing with 3M HCl	1030	-	5
6	Rinsing with water	1030	-	20
7	Conditioning with carrier ampholyte	1030	-	5

2.2.4.2.3. The use of internal standard

The experiment was done with PrinCE Instrument and by following the procedure of Table 7. The use of L-tryptophan and 4-aminobenzoic acid as internal standard was investigated to improve the reproducibility of migration time and peak area.

3. Results and Discussion

3.1. Capillary zone electrophoresis

3.1.1. Analysis of ß-lactoglobulin using PDMAA-coated capillary

As mentioned before, protein adsorption on bare fused-silica capillary using CZE technique is still a major problem in protein analysis. Reversible interactions between the analytes and the silica walls result in tailing, broadening of peaks, and a decrease of reproducibility, whereas irreversible interactions change the surface structure of the capillary. The changes in the surface due to adsorption of proteins result in a different flow profile and EOF in the silica capillaries, further irreproducibility of separation and band broadening occurred. In order to solve this problem, one approach is protein separation at conditions under which the silanol groups are fully coated with polymeric materials. This can control the EOF and minimize the interaction with proteins.

In our experiment, the performance of polydimethylacrylamide (PDMAA)-coated capillary (IMTEK, Albert-Ludwigs Universität Freiburg) was examined with regard to the analysis of proteins. This capillary with i.d. 75 µm was prepared by the polymerization of dimethylacrylamide (DMAA) inside the capillary that is previously coated with a silane having a polymerizable group (MPS, methacryloyloxypropyl trimethoxy silane).

The application of the PDMAA-coated capillaries for protein analysis was performed under variation of the buffer system using CZE. The ability of the capillary coating surface on reducing the interaction with proteins was investigated by evaluating the performance of a PDMAA coating in the protein separations by CE. Therefore, this study was focused on the protein adsorption behavior of the capillary wall. A change of apparent EOF mobility or migration time of the EOF marker can give information about the interaction of proteins and the capillary wall.

The stability of the PDMAA coating was also investigated by measuring the apparent EOF mobility in a long-term use of protein separation under pH conditions close to the p*I* of the protein. In this case, the interactions between protein analytes and polymer coatings affect the stability of PDMAA-coated capillary.

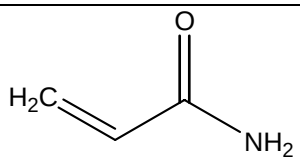
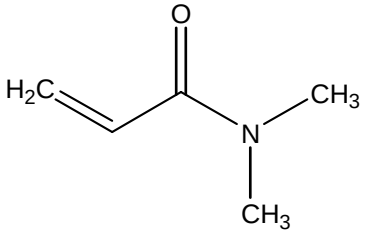
The comparison of PDMAA to bare fused-silica and to LPA-coated capillaries was also discussed. The structure of the monomers used to coat the bare fused-silica capillary,

including acrylamide and *N*,*N*-dimethylacrylamide (DMAA) is shown in Table 8. If proteins were separated using coated capillaries, additional pressure was applied to perform the analysis in a reasonable time frame. Therefore, the apparent EOF is calculated from the measured migration times and is higher than the real occurring EOF. For practical reasons, the apparent EOF was discussed.

In this experiment, ß-lactoglobulin that served as a model protein was separated at different pH values, especially close to its p*I*. Acetanilide that remains uncharged under any applied conditions was chosen as an EOF marker. Neostigmine bromide was used as internal standard, because it provides good stability under any applied conditions.

In order to estimate the precision of protein separation, 30 runs of sample solution containing internal standard and EOF marker were performed, called control 1. The next step was 30 runs of analyte sample solution containing protein, internal standard and EOF marker. Again, 30 runs of sample solution, the same as control 1, were performed to evaluate the performance of capillaries after the protein separation that called control 2. This longer series (n=30) was performed to assure the statistical certainty in order to investigate protein adsorption. RSDs% (relative standard deviation) of migration time, peak area or apparent EOF mobility were used as measurement parameters.

Table 8. Structures of acrylamide and DMAA [53]

Monomer	**Chemical structure**
Acrylamide	
N,*N*-dimethylacrylamide	

3.1.1.1. ß-lactoglobulin analysis at pH 6.5

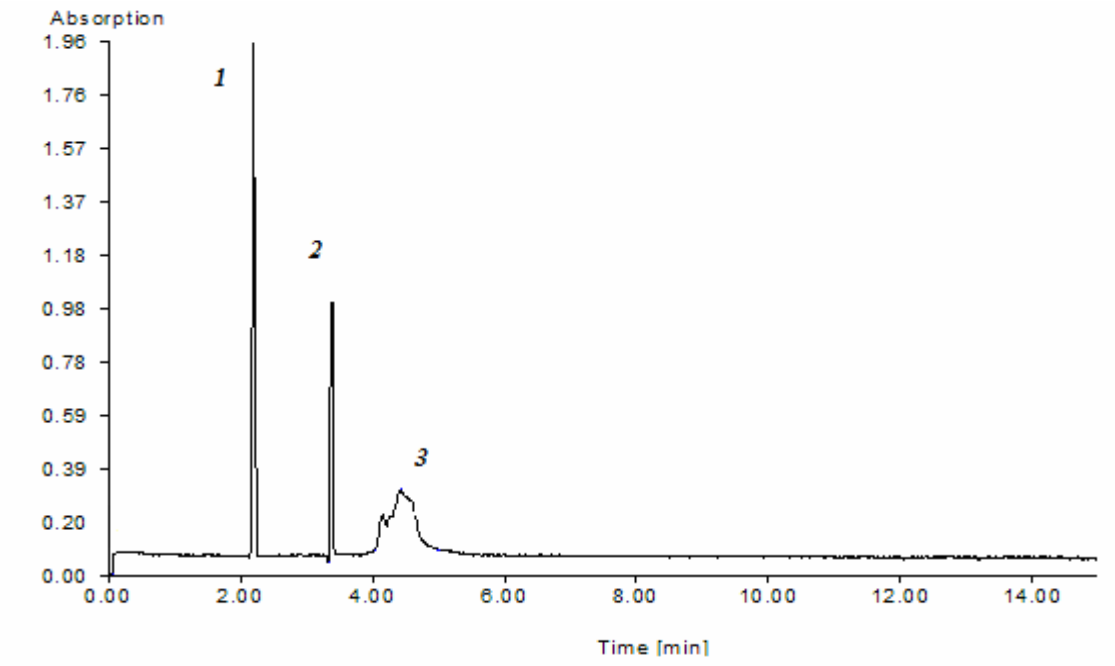


Fig. 16. The electropherogram of protein analysis at pH 6.5 using a bare fused-silica capillary. Peak 1: neostigmine bromide; peak 2: acetanilide; peak 3: ß-lactoglobulin. Phosphate Buffer pH 6.5 (50 mM), V = 25 kV, I ~ 62.4 µA

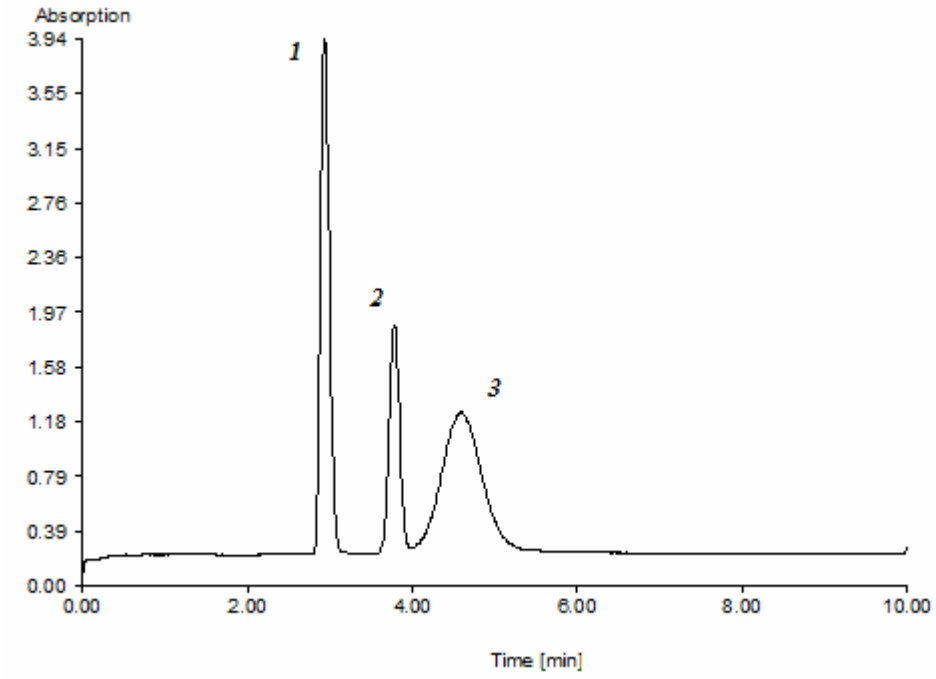


Fig. 17. The electropherogram of protein analysis at pH 6.5 using a PDMAA-coated capillary. Peak 1: neostigmine bromide; peak 2: acetanilide; peak 3: ß-lactoglobulin. Phosphate Buffer pH 6.5 (50 mM), V = 13 kV, P = 40 mbar, I ~ 106 µA

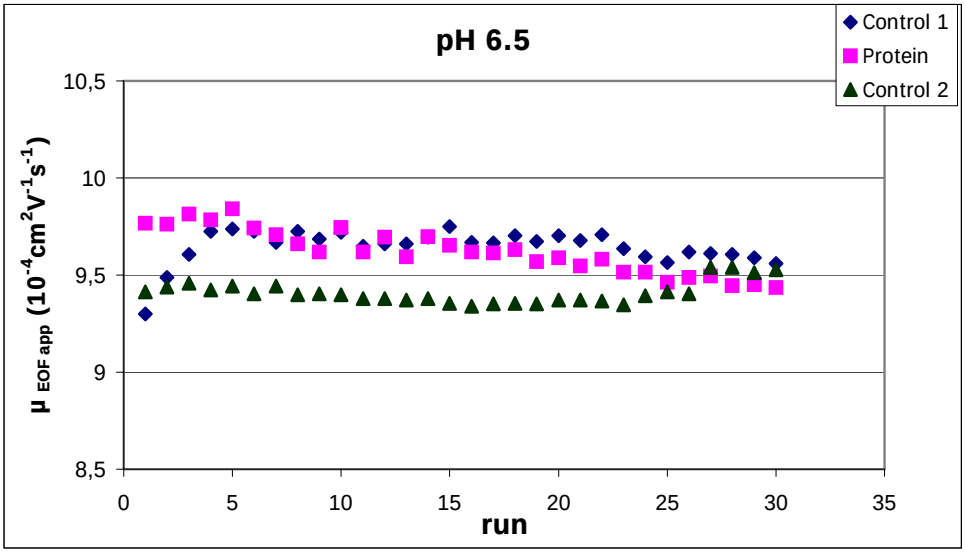


Fig. 18. Apparent EOF mobility at pH 6.5 using a PDMAA-coated capillary (refer to Figure 17)

In order to evaluate the effect of protein adsorption on the capillary wall, the analysis of ß-lactoglobulin that was done at pH 6.5 using bare fused-silica and PDMAA-coated capillaries was discussed. Figure 16 shows the electropherogram of ß-lactoglobulin analysis using bare fused-silica capillary. An asymmetric peak of ß-lactoglobulin that was observed at this pH indicates interactions between the protein and the capillary wall. As discussed before that the protein adsorption on the capillary wall influences the peak shape of protein. Meanwhile, ß-lactoglobulin analysis by the use of PDMAA-coated capillary at the same pH showed a symmetric peak shape (Figure 17). It proved that the usage of PDMAA-coated capillary can prevent protein adsorption on the capillary wall. Preventing protein adsorption is also demonstrated by PDMAA coating with an excellent reproducibility of the apparent EOF mobility (measured by migration time of acetanilide) during the protein analysis, with relative standard deviation (RSD) values of 1.209% for 30 consecutive runs (Figure 18). Based on this result, under the same conditions at this pH, the PDMAA coating can provide better performance of protein separation compare to bare fused-silica capillaries.

3.1.1.2. ß-lactoglobulin analysis at pH 5.5

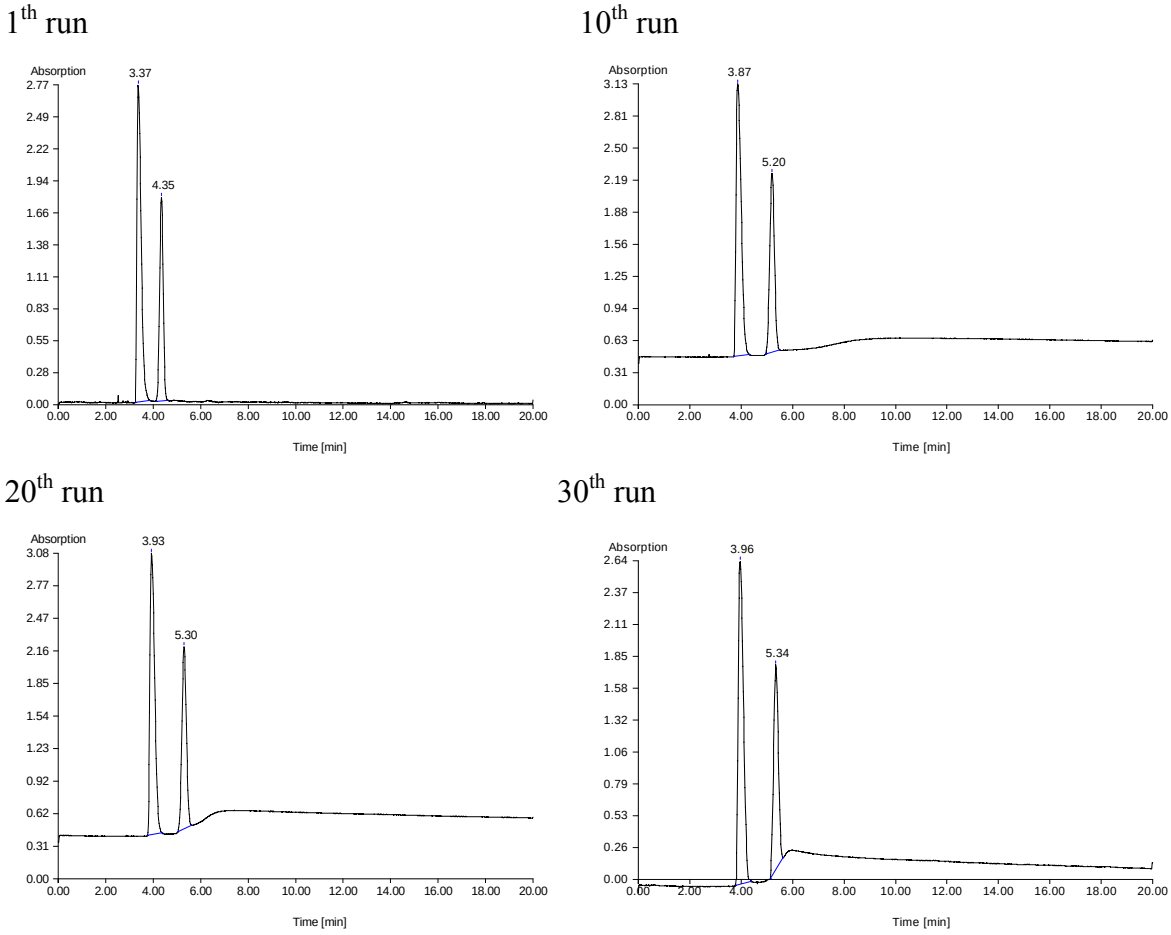


Fig. 19. The electropherogram of protein analysis at pH 5.5 using a PDMAA-coated capillary at the 1th, 10th, 20th, and 30th runs. Peak 1: neostigmine bromide; peak 2: acetanilide. Acetate Buffer pH 5.5 (50 mM), V = 13 kV, P = 40 mbar, I ~ 19 µA

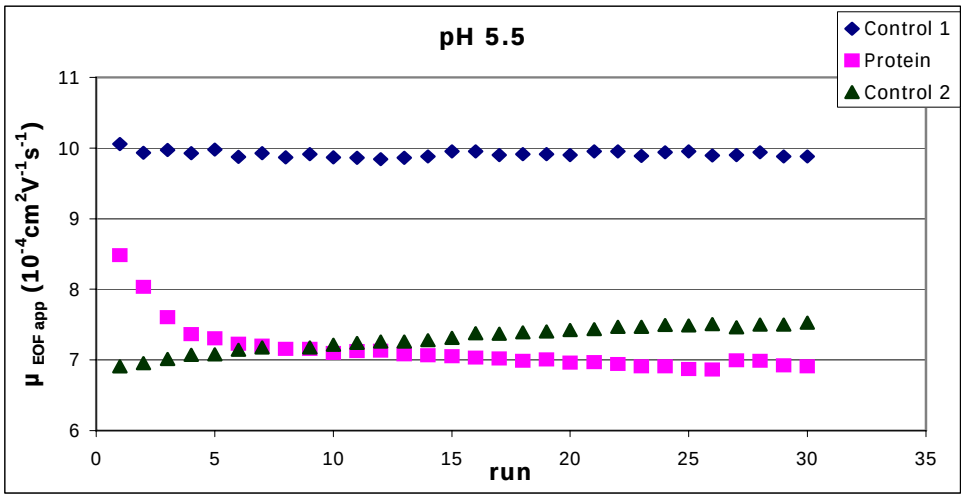


Fig. 20. Apparent EOF mobility at pH 5.5 using a PDMAA-coated capillary (refer to Figure 19)

Figure 19 shows the electropherogram of ß-lactoglobulin analysis at pH 5.5 using PDMAA-coated capillaries at the 1st, 10th, 20th, and 30th runs. The irreproducibility of ß-lactoglobulin peak that indicates the protein adsorption on the capillary wall is observed in this electropherogram. Under this condition, the irreversible adsorption of protein was resulted in runs with no detectable protein peaks at the beginning of series. The adsorbed proteins on the capillary surface may perform aggregation, unfolding or denaturing. This process possibly causes protein aging. After protein aging has been found on the capillary wall, they were more difficult to be removed from capillary surface and completely covered the capillary surface. As a result, ß-lactoglobulin with low recovery was observed after several runs with a un-uniformity in peak shape and migration time.

Based on this result, when PDMAA-coated capillaries are used, strong interactions between positive charges on the protein and negative charges on the wall still take place during electrophoresis at pH 5.5, which this pH is very close to ß-lactoglobulin's p*I*.

The existence of protein adsorption on the capillary wall was also demonstrated by the significant changes in the apparent EOF mobility during protein separation. As shown on Figure 20, the decrease of the apparent EOF mobility was observed since the beginning of series and became much slower after 5th runs. This may indicate that the capillary surface was already completely covered by adsorbed protein. The observed smaller decrease in the following could then be due to ageing processes.

For protein separation at pH 5.5, even the use of PDMAA-coated capillaries was not successful to resolve and detect a protein peak. The irreversibility of protein adsorption could be due to an inhomogeneous coating of the capillary. Some areas of the fused silica that not covered by polymer coating cause protein adsorption. A small thickness of coating layer could be another reason that allows the charges of the silica surface to interact with the proteins on the polymer layer. In general, even though proteins were analyzed using PDMAA-coated capillaries which can efficiently suppress the EOF, the proteins can still be attracted to the surface. This condition influences on the overall separation performance.

3.1.1.3. The comparison of ß-lactoglobulin analysis at different pH

Table 9. RSD (%) of EOF mobility, migration time and peak area using a PDMAA-coated capillary

pH		EOF marker		
		RSD% t_{mig} (min)	RSD% Peak Area	RSD% $\mu_{EOF\ app}$
7.0	Control 1	0.686	1.62	0.684
	Protein	1.801	1.61	1.79
	Control 2	0.970	3.17	0.959
6.5	Control 1	0.944	2.27	0.925
	Protein	1.21	1.36	1.21
	Control 2	0.607	1.023	0.611
6.0	Control 1	4.39	4.13	4.44
	Protein	3.29	3.85	3.303
	Control 2	9.38	9.18	19.8
5.5	Control 1	0.446	3.54	0.448
	Protein	4.35	4.035	4.86
	Control 2	2.52	2.46	2.47

pH	PROTEIN	
	RSD% t_{mig} (min)	RSD% Peak Area
7.0	1.987	4.996
6.5	1.260	2.778
6.0	4.024	4.493
5.5	-	-

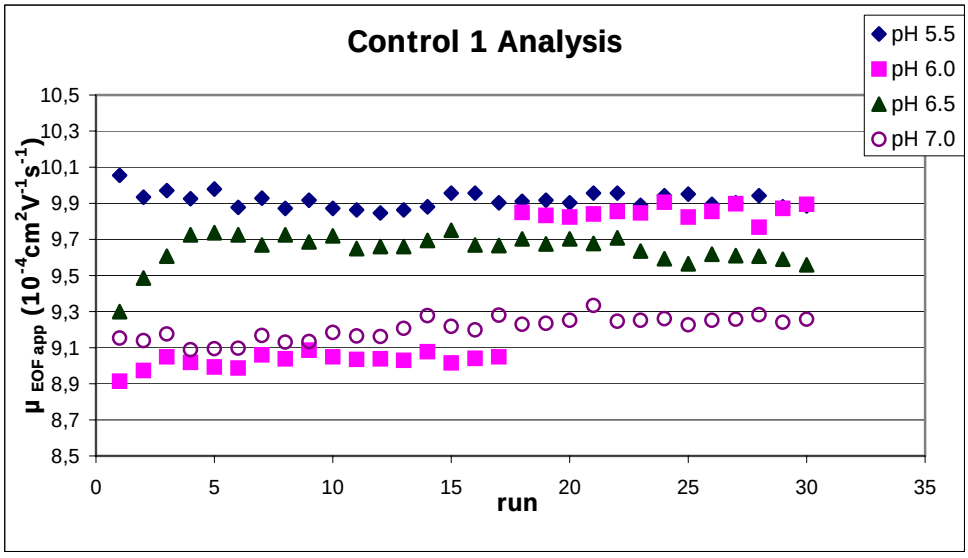


Fig. 21. Apparent EOF mobility of control 1 analysis at each pH using a PDMAA-coated capillary

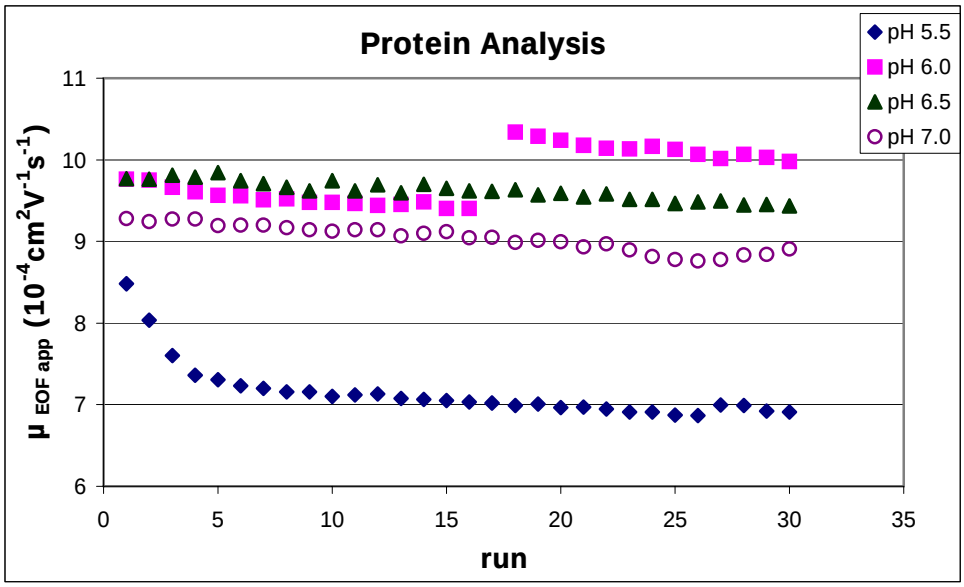


Fig. 22. Apparent EOF mobility of protein analysis at each pH using a PDMAA-coated capillary

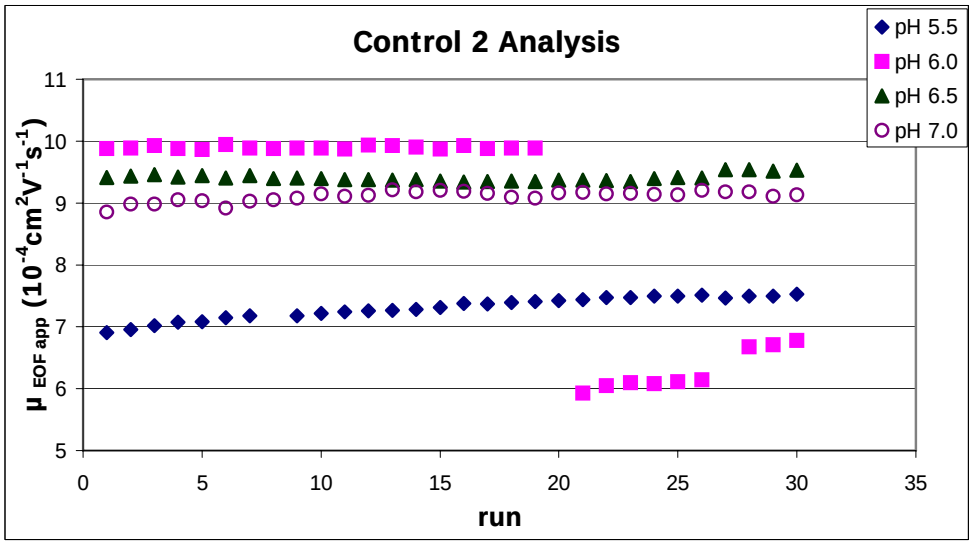


Fig. 23. Apparent EOF mobility of control 2 analysis at each pH using a PDMAA-coated capillary

Using the same PDMAA-coated capillary, firstly, one series of protein analysis was performed at pH 7.0, and then continued with lower pH values respectively. Figure 21-23 and Table 9 shows the reproducibility of migration time and peak area of ß-lactoglobulin and acetanilide and apparent EOF mobility at the different pH values. Within a small pH range close to the p*I* of the protein, the adsorption properties change extremely. At a pH of more than 6.0, the PDMAA coating was much more stable and it was reproducible for 30

consecutive runs, whereas a strong adsorption occurred at pH 5.5. This was proven by the strong decrease of the apparent EOF mobility (Figure 22) and the absence of protein peaks on the protein analysis electropherogram (Figure 19) during the protein analysis at pH 5.5. The jumping line of the apparent EOF mobility after several runs that are observed at pH 6.0 still was not investigated yet. Nevertheless, PDMAA-coated capillaries can be applied to reduce protein adsorption on the capillary wall, and its stability for long-term use proves the high quality of this coating for protein analysis by CZE.

3.1.1.4. The comparison of ß-lactoglobulin analysis on the different types of capillaries

Table 10. RSD t_{mig} (%) of the EOF marker using a bare fused-silica capillary (single use, capillary used only once for one single series) [54]

pH	Control 1	Protein	Control 2	Amount of runs for protein analysis
7.0	1.33	1.19	1.27	30
6.5	1.106	10.57	3.85	30
6.0	9.054	15.8	16.7	30
5.0	1.61	29.8	1.85	30

Table 11. RSD t_{mig} (%) of the EOF marker using a PDMAA-coated capillary (multiple use, one capillary was used for several series at the different pH values)

pH	Control 1	Protein	Control 2	Amount of runs for protein analysis
7.0	0.686	1.801	0.970	30
6.5	0.944	1.208	0.607	30
6.0	4.39	3.85	9.39	30
5.5	0.446	4.35	2.47	30

Table 12. RSD t_{mig} (%) of the EOF marker using a LPA-coated capillary (multiple use) [54]

pH	Control 1	Protein	Control 2	Amount of runs for protein analysis
7.0	3.92	0.973		P_1 : 9 + P_2 : 30
	85.06	14.2		P_1 : 9 + P_2 : 30
6.5	2.64	8.17		30
	1.35	36.7		99
6.0	1.12	18.6		30

Table 13. RSD t_{mig} (%) of the EOF marker using a LPA-coated capillary (single use) [54]

pH	Control 1	Protein	Control 2	Amount of runs for protein analysis
7.0	0.744	13.5	4.49	30
6.5	1.24	0.588	1.37	30
	0.88	0.862	7.48	30
6.08	4.62	9.51	4.13	30
5.25	0.447	2.45	0.882	30

Table 14. Migration time reproducibility (n = 30) of the EOF marker separated in bare fused-silica and polymer-coated capillaries at different pH

pH	Bare fused-silica (single use) [54]		PDMAA-coated capillary (multiple use)		LPA-coated capillary (multiple use) [54]		LPA-coated capillary (single use) [54]	
	Increase in t_{mig} of EOF marker (%)	Adsorption	Increase in t_{mig} of EOF marker	Adsorption	Increase in t_{mig} of EOF marker	Adsorption	Increase in t_{mig} of EOF marker	Adsorption
7.0	< 2	0	< 2	0	<2/(14)	0/(++)	< 14	++
6.5	< 11	+	< 2	0	< 9	+	< 2	0
6.0	< 16	++	< 4	+	< 19	++	< 10	+
5.5			< 5	+				
5.25							< 3	+
5.0	< 30	+++						

In order to evaluate the effectiveness of PDMAA coating for protein analysis, the comparison between bare fused silica and LPA coating performed by Graf was discussed [54]. The peak areas obtained from experiments using the coated capillaries cannot be compared to those obtained by the bare fused-silica capillary even under the same conditions, due to additional pressure that was applied for the polymer-coated capillaries contributes to the analytes velocity. Therefore, the comparison of the migration times, especially those of acetanilide as EOF marker was discussed.

As discussed on subtitle 3.1.1.3., the PDMAA-coated capillaries showed a good stability in a long-term use and can also effectively reduce protein adsorption on the capillary wall. The performance of these capillaries was also compared with bare fused silica and LPA-coated capillaries (Table 10-13).

Systematically, the behavior of the protein adsorption on the capillary wall in the different types of capillaries during the protein analysis (such as bare fused-silica, LPA, and PDMAA-coated capillaries) is shown on Table 14. In general, the LPA and PDMAA-coated capillaries

reduce protein adsorption on the capillary wall compared to bare fused-silica capillaries. It is demonstrated by the excellent reproducibility of migration times of acetanilide during protein separation. It is also proved by the results in which the bare fused-silica capillaries do not provide a good reproducibility at pH below 7.0. Meanwhile, the LPA and PDMAA-coated capillaries even give a better reproducibility at pH 6.0. In case of the comparison between the LPA and the PDMAA-coated capillaries, the PDMAA-coated capillaries show better migration time reproducibility of acetanilide than the LPA-coated capillaries in multiple uses, especially at a pH values close to the p*I* of the protein. When using a new capillary for each series, better results were obtained in general. Therefore, using a new coated capillary for each serie in protein analysis is recommended to provide superior reproducibility. However, the PDMAA and LPA-coated capillaries can decrease but cannot a completely prevent protein adsorption even when using fresh capillaries.

3.1.2. Analysis of ß-lactoglobulin using bare fused-silica capillaries without and with the presence of trehalose or sucrose

As discussed before the stability of a protein may influence its adsorption on the capillary surface. Therefore, the possible way to reduce protein adsorption is, by increasing the conformational stability of proteins and modifying the protein more resistant towards denaturation of the surface. The conformational stability of proteins influences the rate of adsorbed protein that undergoes conformational changes after adsorption. A less stable protein is susceptible to undergo conformational changes from native to denatured state. Each step gives an increased number of protein-surface interactions and between adsorbed proteins themselves [47]. When the aging protein has taken place, it becomes difficult to be removed from the surface. As a result, the protein adsorption becomes irreversibility and it changes the structure of the surface.

Many cases of changing conformational stability of protein during formulation of pharmaceuticals and biotechnological products have been reported. Such at some stage in the preparation, processing, and storage of protein into powders disturb and finally denature them. Many researches use the addition of excipients to protect the protein during spray-drying and lyophilization and improve the dry storage stability by reducing the aggregate formation [50, 55, 56]. In the absence of excipients, spray drying could result in small losses of its enzymatic activity [49].

3.1.2.1. ß-lactoglobulin analysis at pH 6.5: Effect of trehalose

A variety of sugars are known as excipients that have a function to decrease protein adsorption by stabilization of the proteins' native state [47]. Trehalose that is disaccharide consists of two glucose molecules bound by an alpha, alpha-1, 1 linkage with the systematic name α-D-glucopyranosyl α-D-glucopyranoside. This sugar is one of the sugar types that is best known as a good excipient. The structure of trehalose is shown at Figure 24. The addition of trehalose improves the stability and reduces aggregate formation during storage. Trehalose can protect proteins from damage due to dehydration, heat or cold and various stresses, such as dryness, freezing and osmopressure [50, 57].

The behavior of trehalose in enhancing the proteins stability was explained by Lins, 2004 using atomistic molecular dynamic (MD) simulation. As shown in Figure 25, trehalose molecules cluster in the closest part to the protein surface. Nevertheless, it does not completely expel water from the protein surface and also does not form hydrogen bonds with the protein. Trehalose molecules compete with the protein to form hydrogen bonds with the water molecules at the protein surface. Reducing the number of protein-solvent hydrogen bonds will reduce the electrostatic solvation properties on the protein. As a result, the intraprotein interaction is enhanced and a stabilization of the protein native structure is maintained [58].

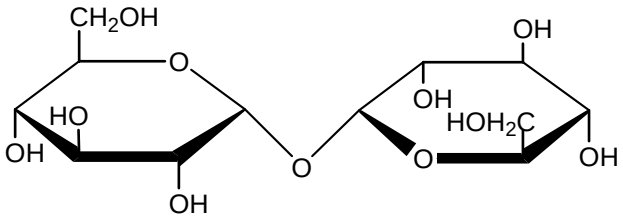


Fig. 24. Structure of trehalose

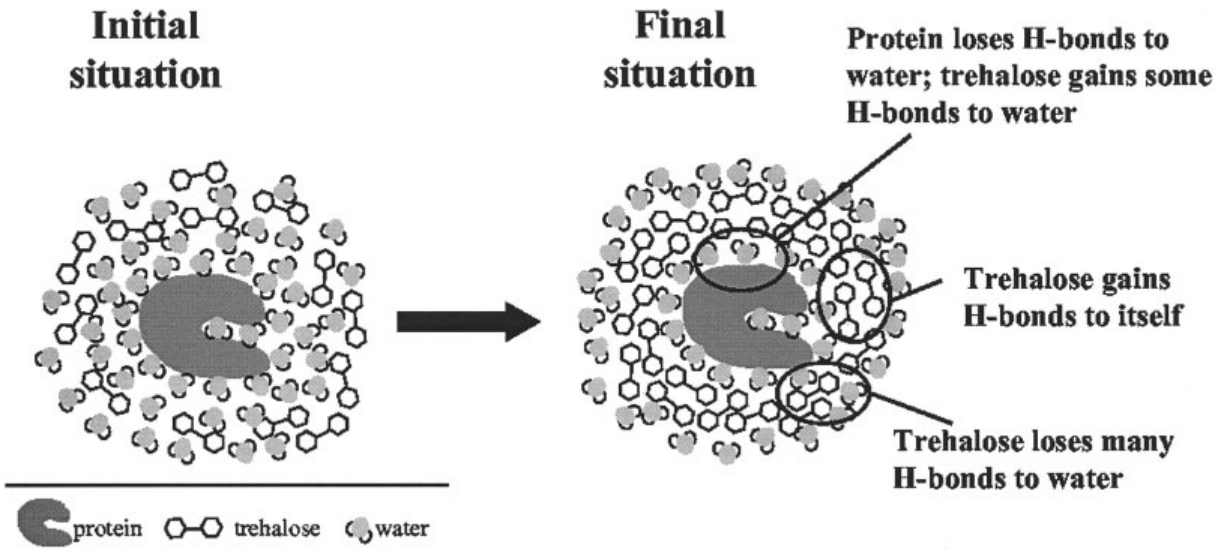


Fig. 25. Interaction of protein and trehalose in aqueous solution [58]

As mentioned before, trehalose as stabilizing reagent has properties such as high thermostability and a wide pH-stability range. The effects of trehalose on protein stability during the protein analysis in CZE were investigated using the bare fused-silica capillaries under pH conditions close to p*I*'s protein.

Figure 26 gives the typical run of ß-lactoglobulin analysis at pH 6.5 using a bare fused-silica capillary in the presence of trehalose 35 µM as protein stabilizer. Compared to the same condition of ß-lactoglobulin analysis without the presence of trehalose (Figure 16), no significant difference in the typical run can be seen. An asymmetric peak of ß-lactoglobulin was still observed at this pH which indicates the interaction between the protein and the capillary wall. The effect of trehalose toward the protein stabilization at this pH may not yet be proven with the measurement of relative standard deviation of the EOF mobility, migration time or peak area (Table 15).

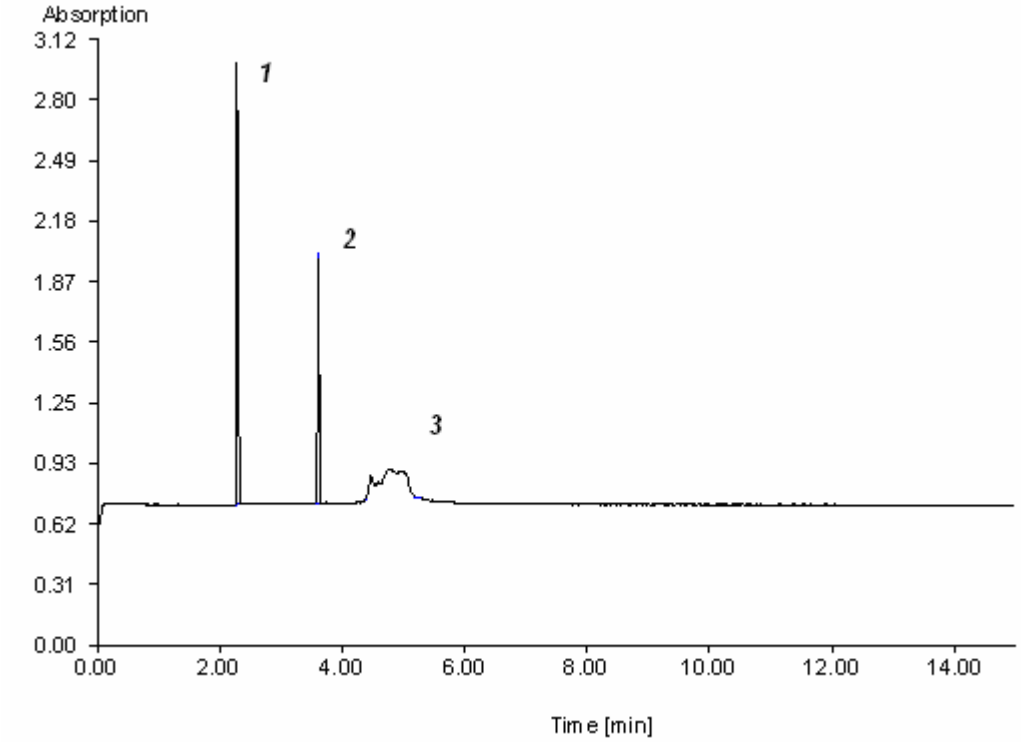


Fig. 26. The electropherogram of protein analysis at pH 6.5 with the presence of trehalose 35 µM using a bare fused-silica capillary. Peak 1: neostigmine bromide; peak 2: acetanilide; peak 3: ß-lactoglobulin. Phosphate Buffer pH 6.5 (50 mM), V = 25 kV, I~ 51 µA

Table 15. RSD (%) of EOF mobility, migration time and peak area at pH 6.5 with the presence of trehalose

pH 6.5		EOF marker			Internal Standard	
		t_{mig}	Peak Area	µ EOF	t_{mig}	Peak Area
without Trehalose	Control 1	1.68	4.22	1.71	1.139	3.86
	Protein	5.007	3.39	4.95	3.482	2.57
	Control 2	0.732	3.032	0.727	0.646	3.22
the presence of Trehalose 35 µM	Control 1	1.63	4.73	1.65	1.264	3.907
	Protein	2.46	2.93	2.48	1.645	2.42
	Control 2	5.52	6.39	5.34	4.243	5.95

pH 6.5	PROTEIN	
	t_{mig}	Peak Area
without Trehalose	5.88	14.5
the presence of Trehalose 35 µM	2.92	4.16

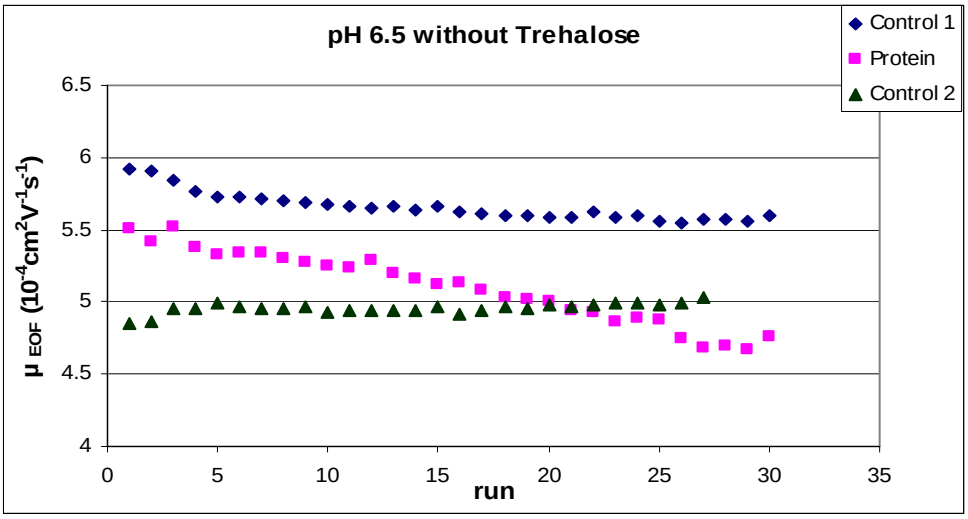


Fig. 27. EOF mobility of ß-lactoglobulin analysis at pH 6.5 without the presence of trehalose using bare fused-silica capillary

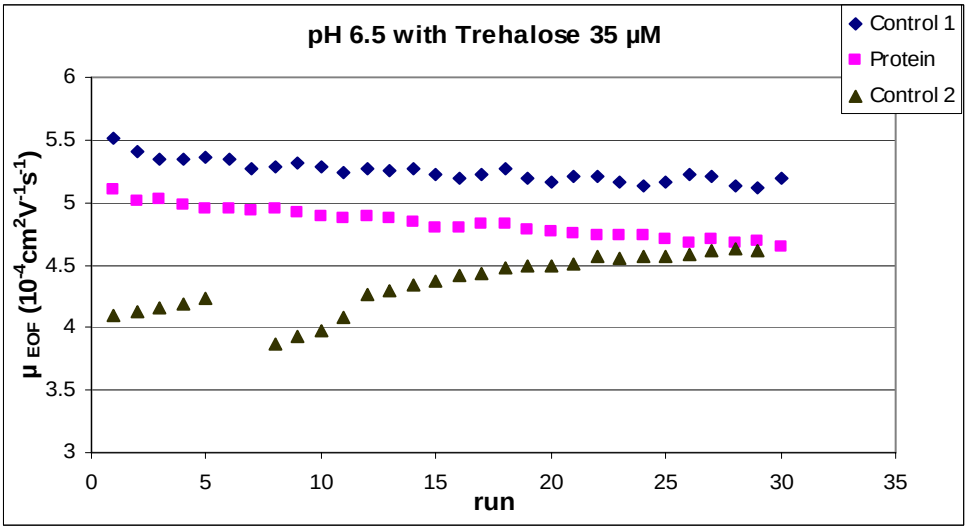


Fig. 28. EOF mobility of ß-lactoglobulin analysis at pH 6.5 with the presence of trehalose 35 μM using a bare fused-silica capillary

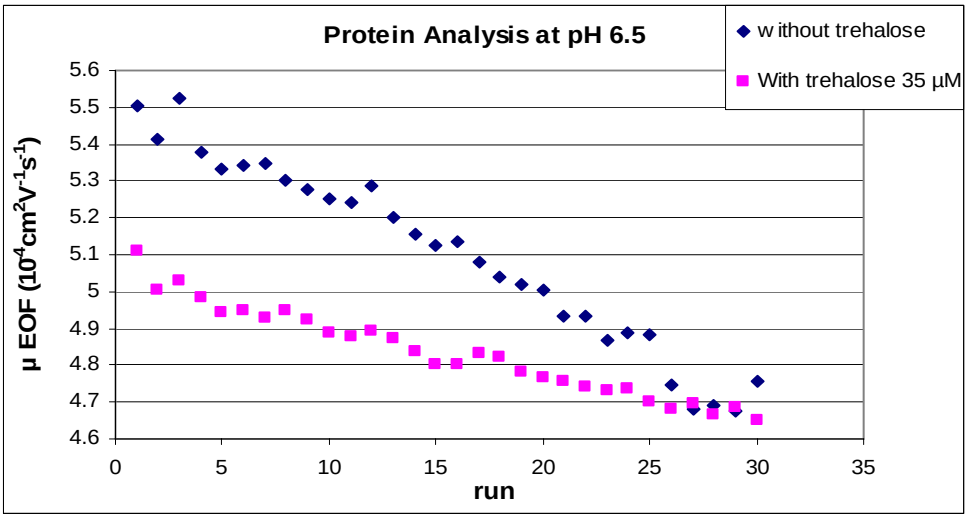


Fig. 29. The comparative of EOF mobility of ß-lactoglobulin analysis at pH 6.5 without and with the presence of trehalose 35 µM using a bare fused-silica capillary

3.1.2.2. ß-lactoglobulin analysis at pH 6.0: Effect of trehalose

Trehalose with concentrations 35 and 70 µM was tested in the ß-lactoglobulin analysis at pH 6.0. The effect of trehalose was evaluated to reduce the protein adsorption by stabilization of the protein as an experiment at pH 6.5. Based on the measurements of relative standard deviation of EOF mobility, migration time or peak area (Table 16), no significant difference between the protein analysis without and with the presence of trehalose is observed, even when a higher concentration of trehalose (70 µM) was used. Sharp decreasing of EOF mobility was observed after several runs (Figure 30). It indicates that the stability of EOF mobility was not achieved by the presence of trehalose in sample and buffer solution.

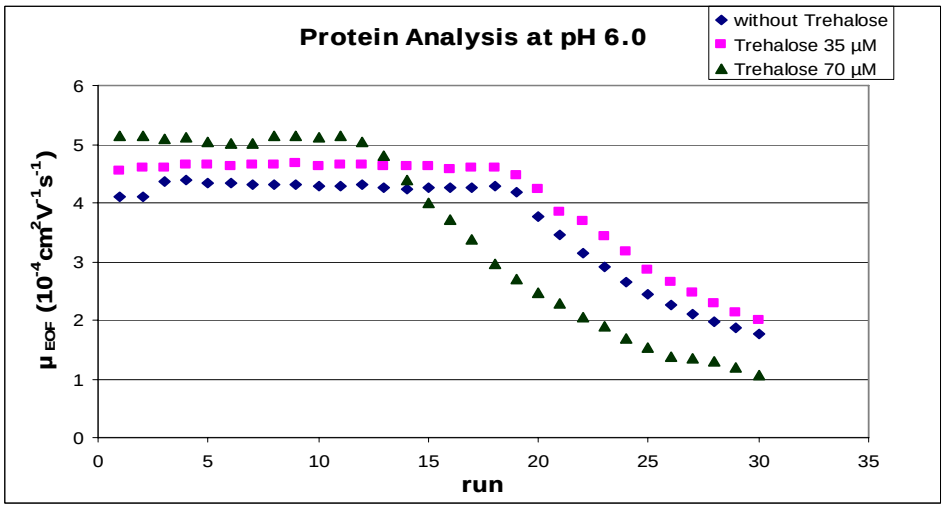


Fig. 30. The comparative of EOF mobility of ß-lactoglobulin analysis at pH 6.0 without and with the presence of trehalose 35 and 70 µM using a bare fused-silica capillary

Table 16. RSD (%) of EOF mobility, migration time and peak area at pH 6.0 with the presence of trehalose

pH 6.0		EOF marker			Internal Standard	
		t_{mig} (min)	Peak Area	μ EOF	t_{mig} (min)	Peak Area
without Trehalose	Control 1	0.72	18.9	0.722	0.866	17.9
	Protein	35.5	38.8	25.3	15.8	18.9
	Control 2	22.7	19.02	31.2	3.69	9.29
with 35μM Trehalose	Control 1	12.6	13.6	11.6	7.32	8.34
	Protein	32.3	40.8	22.9	13.4	22.4
	Control 2	27.3	79.7	38.6	5.085	8.99
with 70μM Trehalose	Control 1	4.036	5.403	3.85	3.28	2.97
	Protein	61.2	95.7	45.5	24.3	29.2
	Control 2	19.1	27.9	19.6	1.79	4.65

pH 6.0	PROTEIN	
	t_{mig} (min)	Peak Area
without Trehalose	63.6	48.3
with 35μM Trehalose	52.8	45.5
with 70μM Trehalose	38.2	45.1

3.1.2.3. ß-lactoglobulin analysis at pH 5.5: Effect of trehalose

The presence of trehalose 35 μM in the sample and acetate buffer solution pH 5.5 was also examined with regard to the effect on the ß-lactoglobulin stabilization. Figure 31 displays the 1st, 10th, 20th and 30th run of a series of successive runs. Starting from the 6th run, the impurity of the acetanilide peak (peak 2a) is detected at the left side of the main peak (peak 2) whereas, in the 1st run, ß-lactoglobulin peak was not detected in the electropherogram. This result shows that the presence of trehalose can not improve the protein analysis that was done in the absence of trehalose under the same condition. This condition was confirmed by the relative standard deviation of EOF mobility, migration time and peak area (Table 17). No obvious difference of the EOF mobility between the absence and the presence of trehalose could be observed (Figure 32). Sharp decrease in the EOF mobility was observed since the 1st run of ß-lactoglobulin analysis with the presence of trehalose with RSD 25.6%. It is different from the EOF mobility which was observed at pH 6.5 and 6.0. A slight decrease of EOF mobility was observed at pH 6.5, and a sharp decrease of EOF mobility was observed after several runs at pH 6.0. In general, based on the result of the experiments that have been prepared with the presence of trehalose at different pH (6.5, 6.0, and 5.5), the presence of trehalose can not be

confirmed to stabilize the protein that expected able to reduce the protein adsorption on the surface of bare fused-silica capillary.

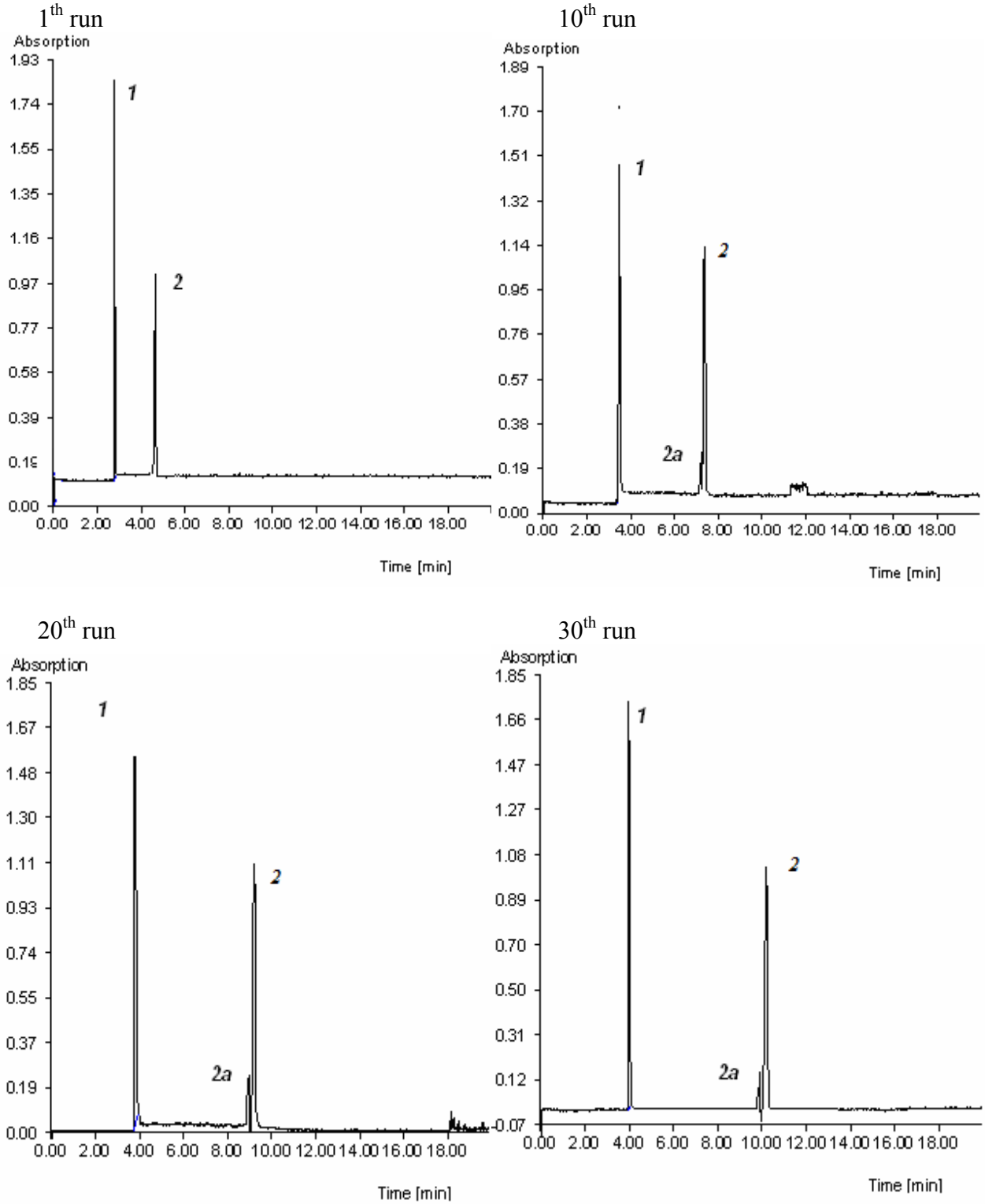


Fig. 31. The electropherogram of protein analysis at pH 5.5 using a bare fused-silica capillary at the 1th, 10th, 20th, and 30th runs. Peak 1: neostigmine bromide; peak 2: acetanilide. Acetate Buffer pH 5.5 (50 mM), V = 25 kV, I~ 34 µA

Table 17. RSD (%) of EOF mobility, migration time and peak area at pH 5.5 with the presence of trehalose

pH 5.5		EOF marker			Internal Standard	
		t_{mig} (min)	Peak Area	μ EOF	t_{mig} (min)	Peak Area
without Trehalose	Control 1	0.626	5.55	0.630	0.690	4.36
	Protein	19.8	18. 9	23.3	10.48	10.82
	Control 2	4.49	4.32	4.47	2.58	4.37
the presence of Trehalose 35 μM	Control 1	1.93	3.62	1.92	1.807	2.81
	Protein	21.02	20.78	25.6	9.98	12.07
	Control 2	0.859	3.109	0.849	0.610	5.055

pH 5.5	PROTEIN	
	t_{mig} (min)	Peak Area
without Trehalose	-	-
the presence of Trehalose 35 μM	-	-

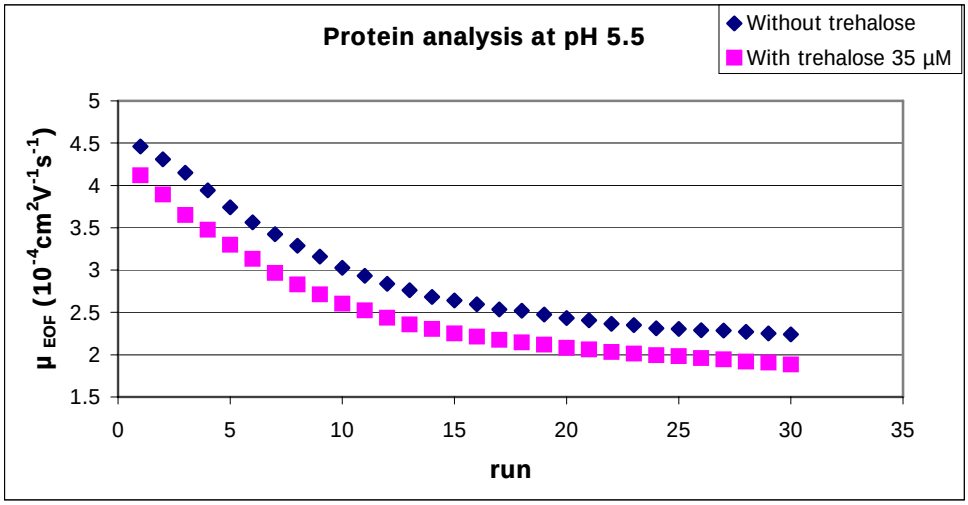


Fig. 32. The comparative of EOF mobility of ß-lactoglobulin analysis at pH 5.5 without and with the presence of trehalose 35 μM using a bare fused-silica capillary

3.1.2.4. ß-lactoglobulin analysis at pH 6.0: Effect of sucrose

Although the presence of trehalose was not successful to stabilize the protein, another variety of sugar like sucrose was considered. Sucrose is a disaccharide of glucose and fructose with systematic name α-D-glucopyranosyl-β-D-fructofuranoside, its structure is shown in Figure 33. This consideration is based on the experiment with the presence of sucrose that was performed by Tzannis (1999). The presence of sucrose was successful to preserve structure and activity of trypsinogen as a thermal and dehydration stress stabilizer. Complete activity in preservation was achieved even at low concentrations of sucrose [49]. Kim also proved the effects of sucrose on conformational equilibria within the native-state of ß-lactoglobulin.

Beside sucrose can increase the thermodynamic conformational stability of proteins, it also shifts the equilibrium between native and denatured state to favor the native state and inhibit the formation of aggregation states [55].

As the experiment with the presence of trehalose, the effect of sucrose on the stability of proteins during the protein analysis in CZE was investigated by using the bare fused-silica capillaries at pH 6.0. ß-lactoglobulin was used as a model protein in this experiment. As shown at Table 18 and Figure 34, no difference between the absence and presence of sucrose was observed. The sharp decrease of EOF mobility was observed after several runs with RSD 22.9%.

In general, no significant difference of the reproducibility of EOF mobility was observed between the presence and the absence of trehalose and sucrose during protein analysis. It indicates that the suggested influence of trehalose and sucrose in reducing protein adsorption could not be confirmed.

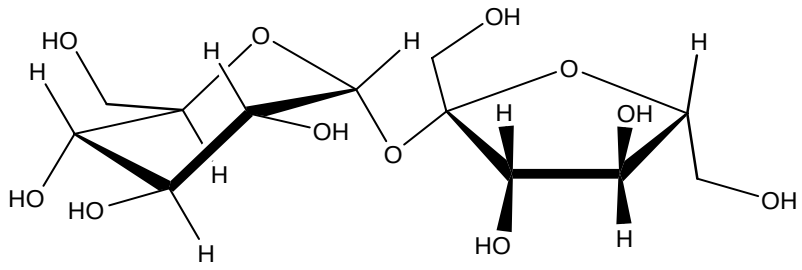


Fig. 33. Structure of sucrose

Table 18. RSD (%) of EOF mobility, migration time and peak area at pH 6.0 with the presence of sucrose

pH 6.0		**EOF marker**			**Internal Standard**	
		t_{mig} (min)	**Peak Area**	**μ EOF**	**t_{mig} (min)**	**Peak Area**
without Sucrose	Control 1	0.720	18.9	0.722	0.866	17.9
	Protein	35.5	38.8	25.3	15.8	18.9
	Control 2	22.7	19.02	31.2	3.69	9.29
the presence of Sucrose 35 μM	Control 1	1.69	2.98	1.709	1.18	2.43
	Protein	32.3	39.4	22.9	13.3	17.3
	Control 2	23.4	20.7	29.9	4.060	11.7

pH 6.0	**PROTEIN**	
	t_{mig} (min)	**Peak Area**
without Sucrose	63.6	48.3
the presence of Sucrose 35 μM	51.1	29.1

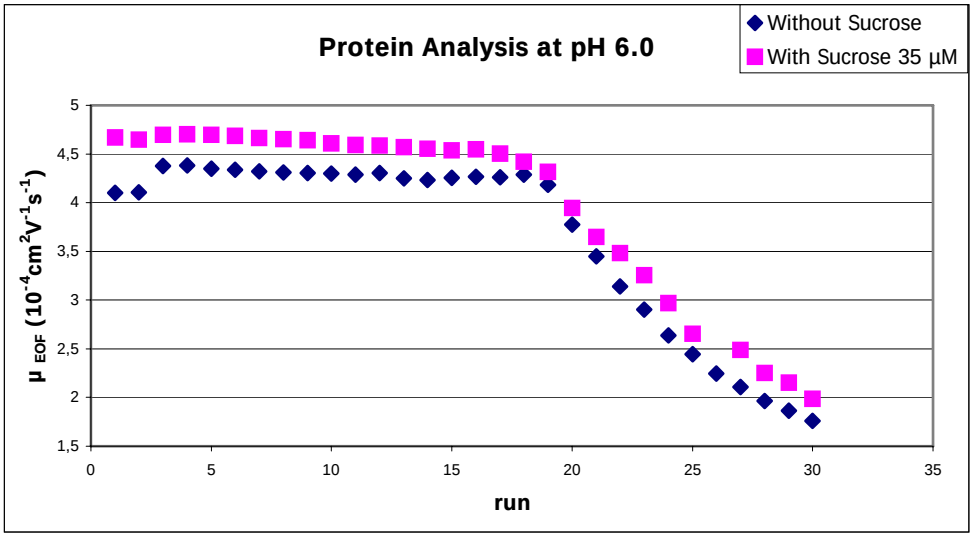


Fig. 34. The comparative of EOF mobility of ß-lactoglobulin analysis at pH 6.0 without and with the presence of sucrose 35 µM using a bare fused-silica capillary

3.1.2.5. The regeneration of capillary

Washing the capillary after protein separation seems to be efficient to release proteins accumulated on the capillary wall. Many researches used SDS to regenerate the capillaries for removing the adsorbed protein with the form of SDS micelles [45, 59]. Nevertheless, capillary rinsing with the buffers containing SDS was successful only for the freshly adsorbed protein [45]. Related to this reason, the rinsing solution that contents of NaOH 1 mol/L, 2-propanol 10% and SDS 200 mmol/L was used in this experiment to regenerate capillary after one series of ß.lactoglobulin separation at pH 6.0. As shown in Table 19 and Figure 35, the similar RSD% of EOF mobility before and after regeneration in the presence of trehalose 70 µM was observed with 28.6% and 29.7%, respectively, whereas the RSD% of the EOF mobility before and after regeneration in the presence of sucrose 35 µM was observed with 22.9% and 26.4%, respectively (Table 20 and Figure 36). This result indicates that the stability of the capillary can be maintained by the rinsing with SDS solution after protein separation.

Table 19. RSD (%) of EOF mobility, migration time and peak area at pH 6.0 with the presence of trehalose (70 µM); before and after regeneration

pH 6.0		EOF marker			Internal Standard	
		t_{mig} (min)	Peak Area	µ EOF	t_{mig} (min)	Peak Area
before regeneration	Control 1	1.84	3.79	1.86	1.09	3.018
	Protein	41.6	45.2	28.6	15.8	21.5
	Control 2	21.6	16.7	28.8	3.64	8.21
after regeneration	Control 1	1.702	3.708	1.72	1.45	2.204
	Protein	41.4	47.4	29.7	17.6	23.1
	Control 2	22.5	31.2	27.5	12.4	61.7

pH 6.0	PROTEIN	
	t_{mig} (min)	Peak Area
before regeneration	50.52	52.7
after regeneration	49.6	35.8

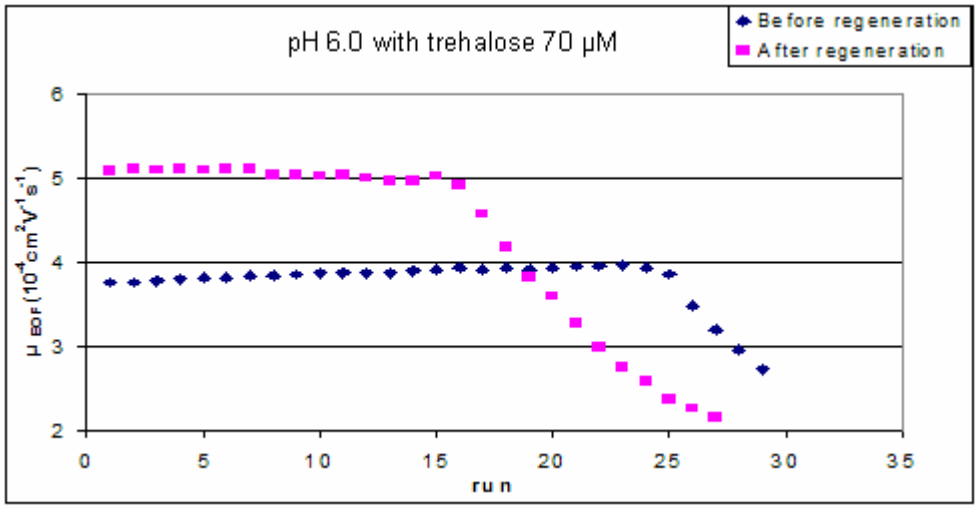


Fig. 35. The comparison of EOF mobility of ß-lactoglobulin analysis at pH 6.0 with the presence of trehalose 70 µM before and after regeneration of a bare fused-silica capillary

Table 20. RSD (%) of EOF mobility, migration time and peak area at pH 6.0 with the presence of sucrose (35 µM); before and after regeneration

pH 6.0		EOF marker			Internal Standard	
		t_{mig}	Peak Area	µ EOF	t_{mig}	Peak Area
before regeneration	Control 1	1.69	2.98	1.709	1.18	2.43
	Protein	32.3	39.4	22.9	13.3	17.3
	Control 2	23.4	20.7	29.9	4.060	11.7
after regeneration	Control 1	1.23	2.53	1.22	0.936	4.44
	Protein	37.2	44.2	26.4	16.1	19.2
	Control 2	31.2	21.2	37.4	12.6	25.5

pH 6.0	PROTEIN	
	t_{mig}	Peak Area
before regeneration	51.1	29.1
after regeneration	54.2	53.5

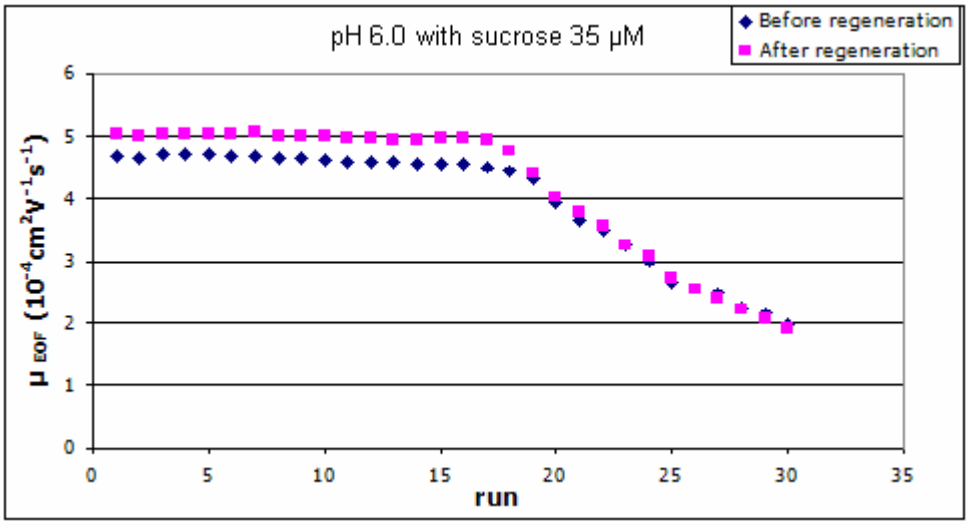


Fig. 36. The comparison of EOF mobility of ß-lactoglobulin analysis at pH 6.0 with the presence of sucrose 35 µM before and after regeneration of a bare fused-silica capillary

3.1.3. Analysis of ß-lactoglobulin, cytochrome c and ß-casein using bare fused-silica capillaries with the presence of PEG as a buffer additive

The common technique used to inhibit protein adsorption is blocking the adsorption sites by the optimization of the molecule (solute) surfaces or/and the coating solid surfaces. The design and preparation of both methods should be suitable for the purpose in many medical or biotechnological applications. In order to achieve solute surface that resist the proteins adsorption from aqueous solution, the exclusion of solute from the protein surface in aqueous

solution is presented, and then the stabilization of the native structure of proteins was achieved [60].

The preferential exclusion of solute from protein surface was presented at Fig. 12a. The solute can be considered into two domains: a local domain that exist in the vicinity of the protein surface and a bulk domain. If the concentration of the solute in the local domain is lower than in the bulk solution, an effective preferential exclusion of the solute from the protein surface occurs. Under this condition, hydrated protein is preferable. Solute that is well-excluded from the protein surface offers a good protein-resistant surface. These solutes having ability to provide "protein resistance" are called kosmotropes. In fact the kosmotropes molecules do not interact directly with the proteins lead to a stabilization of native proteins. Many kosmotrope substances, such as sucrose, maltose, mannitol, taurine, betaine, PEG, DMA, DMSO, and HMPA are effective in the exclusion from the protein surfaces [60, 61].

The second way to block the adsorption site is by coating of the solid surface. Many methods have been used to coat the solid surface. The simplest one can be achieved by the formation of self-assembled monolayers (SAMs). Nevertheless, the effective blocking on adsorption sites is achieved with the formation of SAMs based on displays of kosmotropes. As shown at Figure 12b, they can form a layer on a solid surface and also keep a water layer between protein and SAMs [60, 62]. Many self-assembly systems based on displays of kosmotropes have been investigated to resist protein adsorption, such as derivatives of carbohydrates, alkanethiolates and poly(ethylene glycol) (PEG) [60, 63-68].

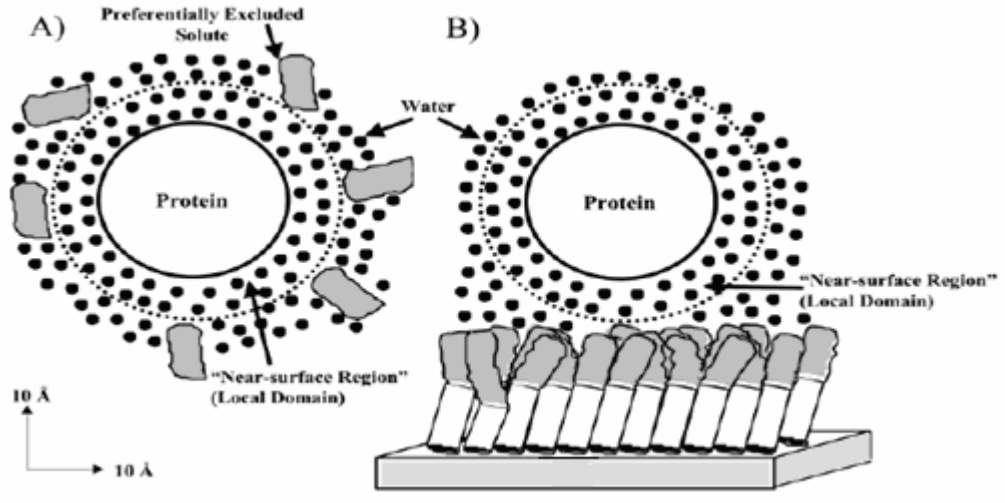
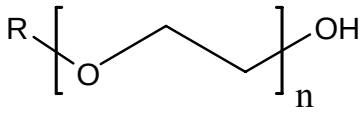


Fig. 12. Schematic representation of a) preferential exclusion solute in the mixture of water and proteins and b) SAMs formation on the solid surface [60]



$R = CH_2CH_2OH$

Fig. 13. Poly(ethylene glycol) structure

Poly(ethylene glycol) is a simple main-chain polyether with the structure shown in Figure 13. It is water soluble, neutral, non-toxic, highly mobile in aqueous solutions, serves as kosmotrope substance and as an excellent coating material. Based on its properties, PEG is among the most commonly used to resist protein binding.

In this experiment, ß-lactoglobulin, cytochrome c, and ß-casein as model proteins were separated by CZE in a bare fused-silica capillary. The molecular mass of these proteins is 11.7-24 kDa with p*I*s of 4.63-9.59. It means that there is strong adsorption of the proteins onto the capillary at a pH around their p*I*s. PEG was evaluated as an additive to avoid the protein adsorption on this capillary. Different concentrations of PEG (3.2 mg/mL and 32 mg/mL) were added into the running buffer solution. Since the addition of PEG 32 mg/mL into the buffer solution causes an increasing viscosity of the solution, an additional pressure of 100 mbar was applied during protein separation in order to reduce the analysis time. Hence, the apparent of EOF mobility in this experiment was used as measurement parameter.

As shown in Figure 14, ß-lactoglobulin which was analyzed without the presence of PEG in the phosphate buffer pH 6.0 showed a decreasing EOF mobility after 18th runs with an RSD of 25.3% (Table 21). It indicated that the formation of protein-layers on the bare fused-silica surface occurred in this series. It changes the surface structure of the capillary wall, and then influences the EOF mobility.

In order to avoid the formation of protein-coated capillaries, PEG 3.2 mg/mL was evaluated under the same condition. A slight difference of apparent EOF mobility was observed between the absence of PEG and the presence of PEG 3.2 mg/mL. The slightly increasing reproducibility of apparent EOF mobility was observed in Figure 15 with an RSD of 17.3% (Table 21). The interaction between protein and capillary wall was not completely avoided by the addition of PEG 3.2 mg/mL.

Subsequently, an increasing concentration of PEG to 32 mg/mL was also evaluated under phosphate buffer pH 6.0. Figure 16 shows the electropherogram of ß-lactoglobulin analysis in the 1st, 10th, 20th, and 30th runs at pH 6.0. The migration time of the internal standard and the

EOF marker were highly reproducible. Nevertheless, a shift in the ß-lactoglobulin migration toward faster analysis time occurred. ß-lactoglobulin might probably not be stable at this pH due to the long-term analysis that might change in protein surface. The number of negative charges of ß-lactoglobulin decreased. It causes a faster mobility. Nevertheless, the formation of protein layer on the capillary was not found in this experiment. This was shown by an excellent reproducibility of apparent EOF mobility with RSD 0.611% (Figure 17 and Table 21). Interactions between ß-lactoglobulin and capillary wall could therefore be prevented by the addition of PEG with a concentration of 32 mg/mL.

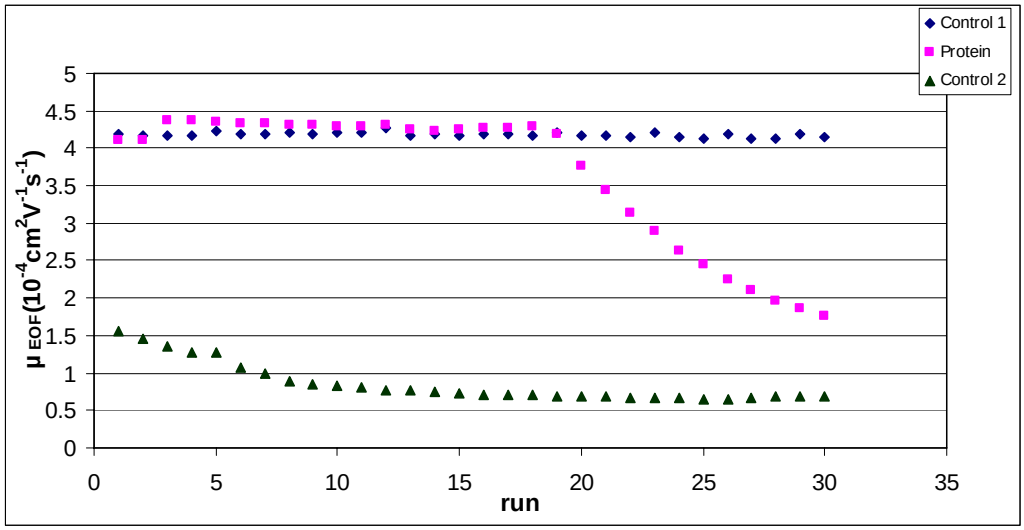


Fig. 14. EOF mobility of ß-lactoglobulin analysis at pH 6.0 without the presence of PEG using a bare fused-silica capillary

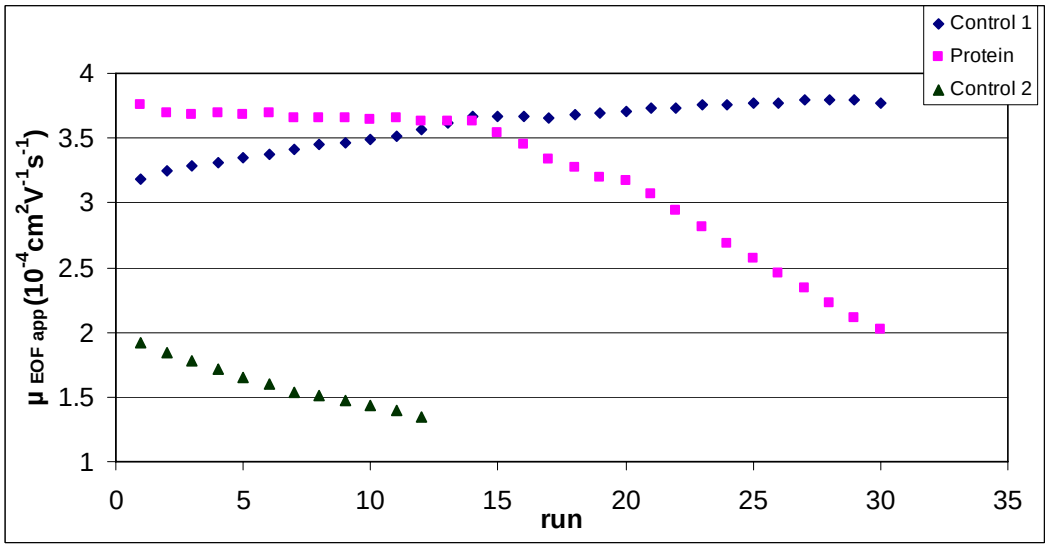


Fig. 15. Apparent EOF mobility of ß-lactoglobulin analysis at pH 6.0 with the presence of PEG 3.2 mg/mL using a bare fused-silica capillary

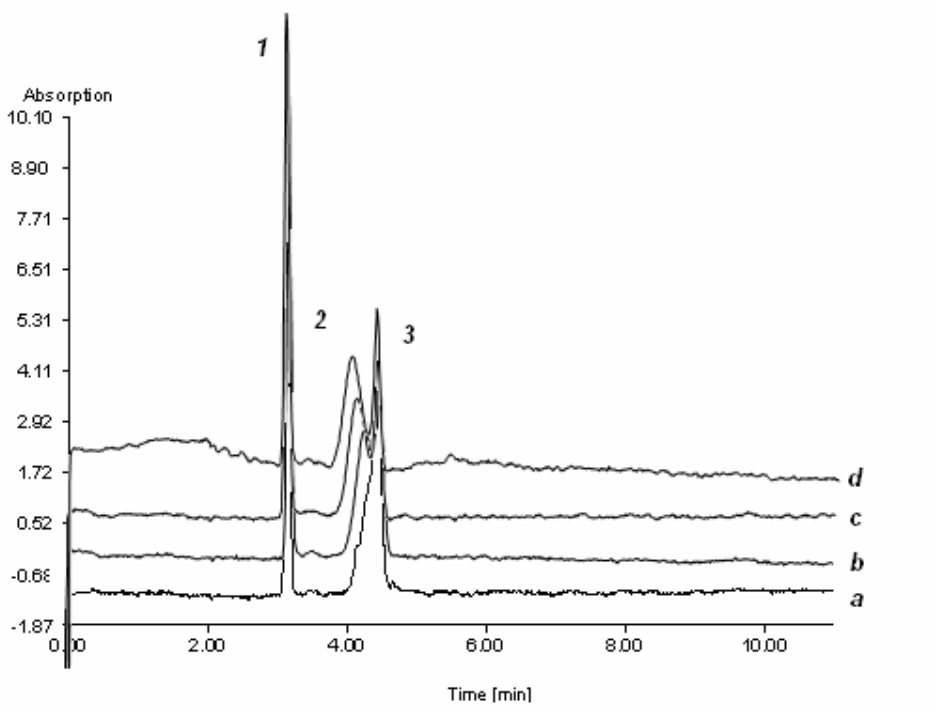


Fig. 16. The electropherogram of ß-lactoglobulin analysis at the (a) 1st (b) 10th (c) 20th (d) 30th at pH 6.0 with the presence of PEG 32 mg/mL using a bare fused-silica capillary. Peak 1: neostigmine bromide; peak 2: ß-lactoglobulin; peak 3: acetanilide. Phosphate buffer pH 6.0 (50 mM), V = 18 kV, I~ 75 µA, additional pressure: 100 mbar

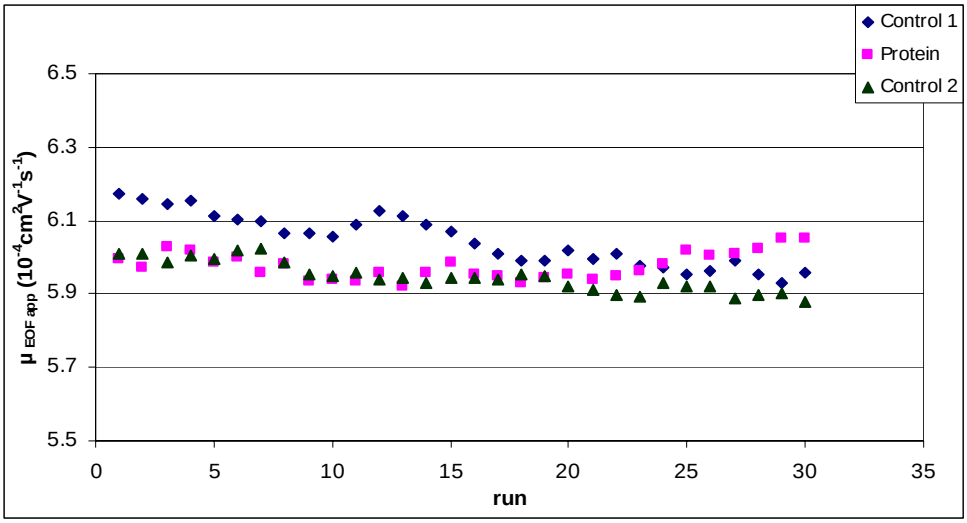


Fig. 17. Apparent EOF mobility of ß-lactoglobulin analysis at pH 6.0 with the presence of PEG 32 mg/mL using a bare fused-silica capillary

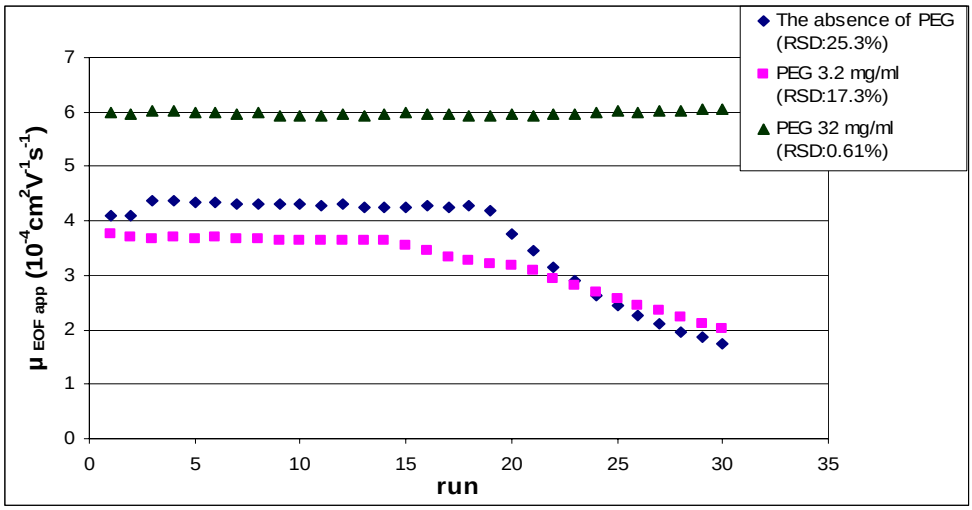


Fig. 18. Apparent EOF mobility of ß-lactoglobulin analysis at pH 6.0 in the absence and the presence of PEG 3.2 and 32 mg/mL

The benefit of the presence of PEG 32 mg/mL was also investigated at the different pH values, especially at the pH close to protein's p*I*. The possibility of protein interaction on capillary wall becomes higher at a pH lower than protein's p*I*. ß-lactoglobulin was analyzed at 50 mmol/L acetate buffer pH 5.0. It was performed after one series of ß-lactoglobulin analysis at pH 6.0 by using of the same capillary. Before this capillary was used for next experiment, it was reconditioned by the same manner as new capillary. It was previously conditioned with 1 mol/L NaOH for 2 hours, continued by a rinsing with buffer for 30 minutes (1200 mbar), then equilibrated for 2 hours with the applied voltage and was afterwards directly used for protein analysis at pH 5.0. Surprisingly, a good stability of internal standard, ß-lactoglobulin and EOF marker was observed, even overlapping between ß-lactoglobulin and EOF marker peak was observed (Figure 19). The successful use of PEG in this experiment is also confirmed by an excellent reproducibility of apparent EOF mobility with RSD 1.2 %.

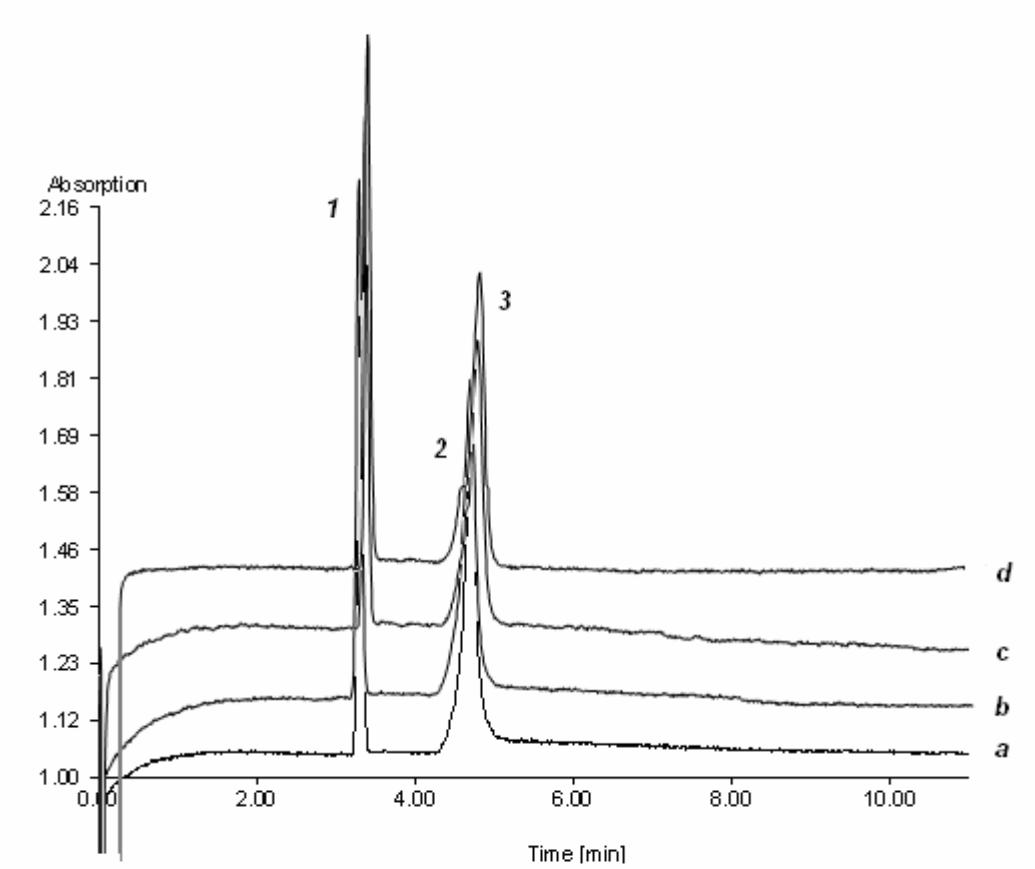


Fig. 19. The electropherogram of ß-lactoglobulin analysis at the (a) 1st (b) 10th (c) 20th (d) 30th at pH 5.0 with the presence of PEG 32 mg/mL using a bare fused-silica capillary. Peak 1: neostigmine bromide; peak 2: ß-lactoglobulin; peak 3: acetanilide. Acetate buffer pH 5.0 (50 mM), V = 18 kV, I ~ 72 µA, additional pressure: 100 mbar

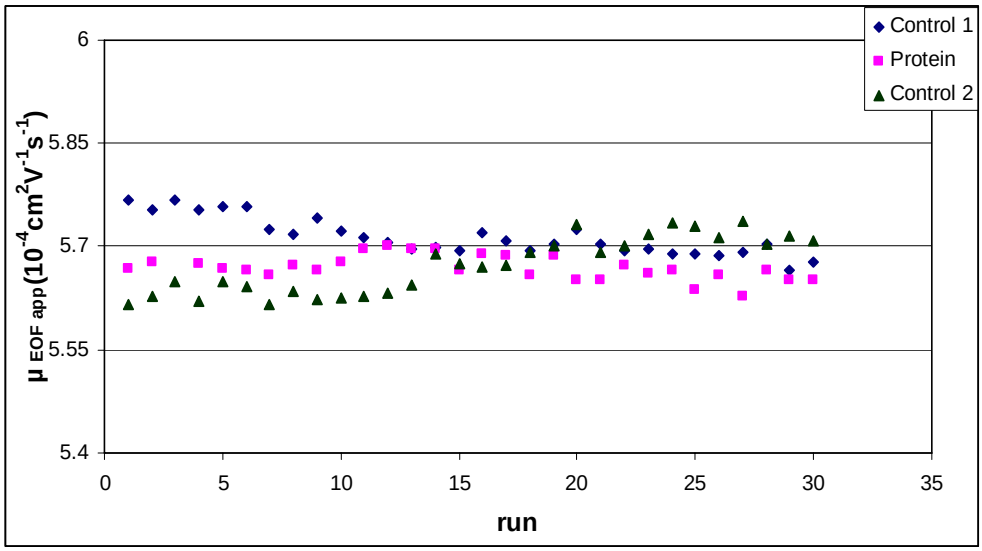


Fig. 20. Apparent EOF mobility of ß-lactoglobulin analysis at pH 5.0 in the presence of PEG 32 mg/mL using a bare fused-silica capillary

In order to confirm the benefit of PEG to resist protein adsorptions, ß-lactoglobulin as an acidic protein, cytochrome c as a basic protein and ß-casein as a more easily denaturing protein were investigated in 50 mmol/L acetate buffer pH 4.0. As shown in the electropherogram (Figure 21), the analysis of ß-lactoglobulin at pH 4.0 shows an optimal condition of analysis. A good stability of migration time and a good resolution of each analyte peak were observed at pH 4.0. The excellent reproducibility of apparent EOF mobility was observed with RSD 0.5% (Figure 24, Table 21).

As cytochrome c is a basic protein with p*I* 9.59, the analysis of cytochrome c using bare fused-silica capillary with the absence of PEG shows strong adsorptions at a pH below 10.5 [45]. However, with the addition of 32 mg/mL PEG into buffer solution, the protein adsorption on capillary wall can be suppressed. This was proven by the electropherogram that showed the stability of the migration time of each analyte peak, even though the resolution was not so good (Figure 22). Protein adsorption on the capillary wall was not found in this experiment which was confirmed by the reproducibility of the apparent EOF mobility (RSD 0.41%, Table 22).

The effectiveness of 32 mg/mL PEG to resist protein adsorption was also evaluated for ß-casein (p*I* 4.6) analysis at pH 4.0. ß-casein analysis at pH 4.0 even with the addition of PEG into buffer solution was not successful in resolving and detecting a protein peak. It was probably due to the fact that ß-casein undergoes the conformational structure change at pH 4.0. Nevertheless, the apparent EOF mobility remains stable with RSD 1.05% (Table 23).

In general, the presence of 32 mg/mL PEG 20000 to 50 mmol/L phosphate buffer pH 6.0 and acetate buffer pH 5.0 and 4.0 has proven to be an effective way to suppress protein adsorption in long-term measurement.

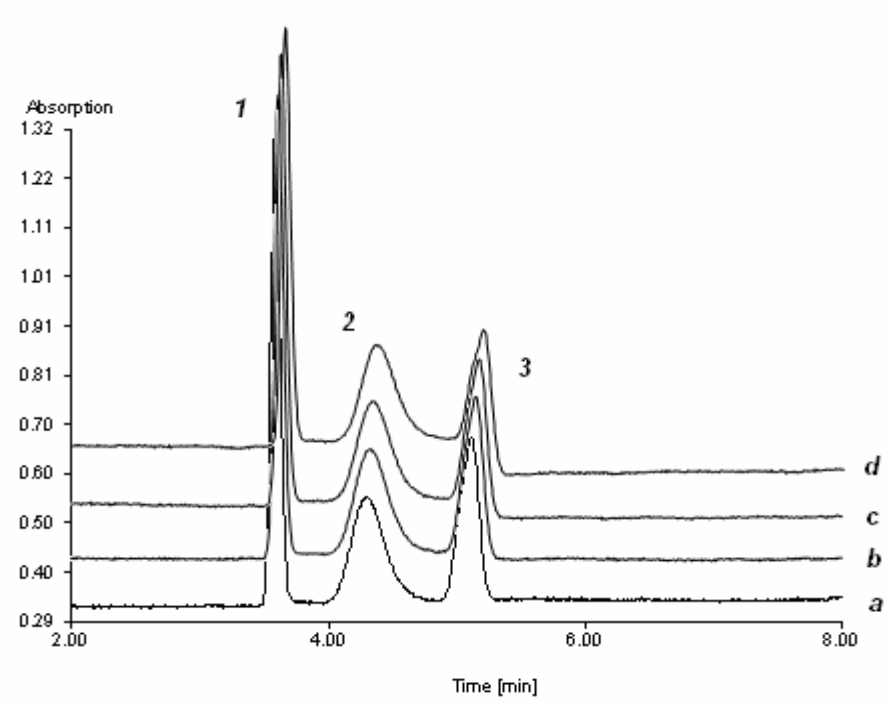


Fig. 21. The electropherogram of ß-lactoglobulin analysis at (a) 1st (b) 10th (c) 20th (d) 30th at pH 4.0 with the presence of PEG 32 mg/mL using a bare fused-silica capillary. Peak 1: neostigmine bromide; peak 2: ß-lactoglobulin; peak 3: acetanilide. Acetate buffer pH 4.0 (50 mM), V = 18 kV, I ~ 46 µA, additional pressure: 100 mbar

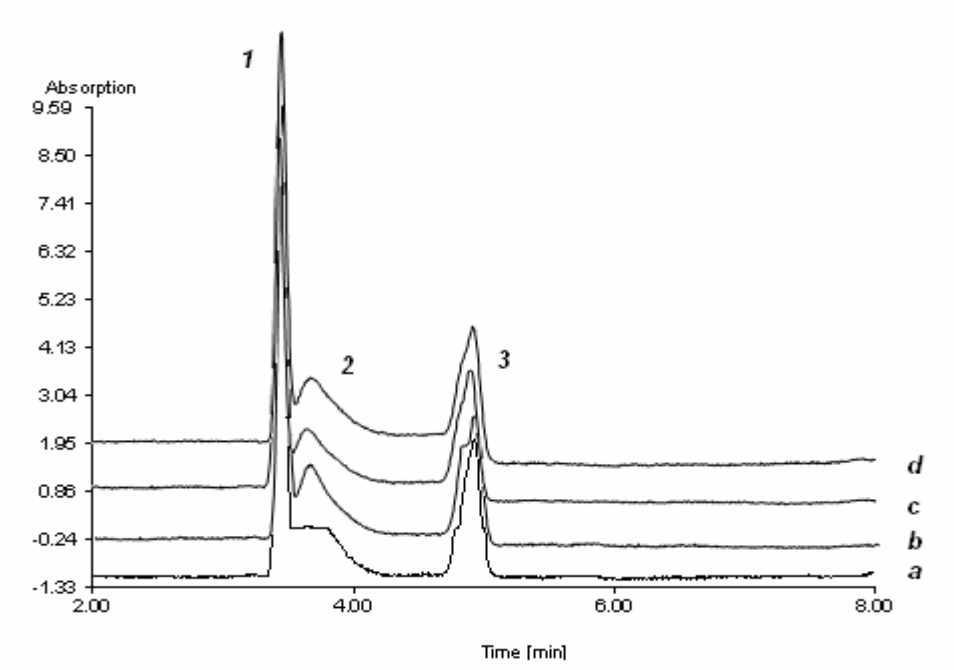


Fig. 22. The electropherogram of cytochrome c analysis pH 4.0 with the presence of PEG 32 mg/mL using a bare fused-silica capillary. Peak 1: neostigmine bromide; peak 2:cytochrome c; peak 3: acetanilide. Acetate buffer pH 4.0 (50 mM), V = 18 kV, I ~ 46 µA, additional pressure: 100 mbar

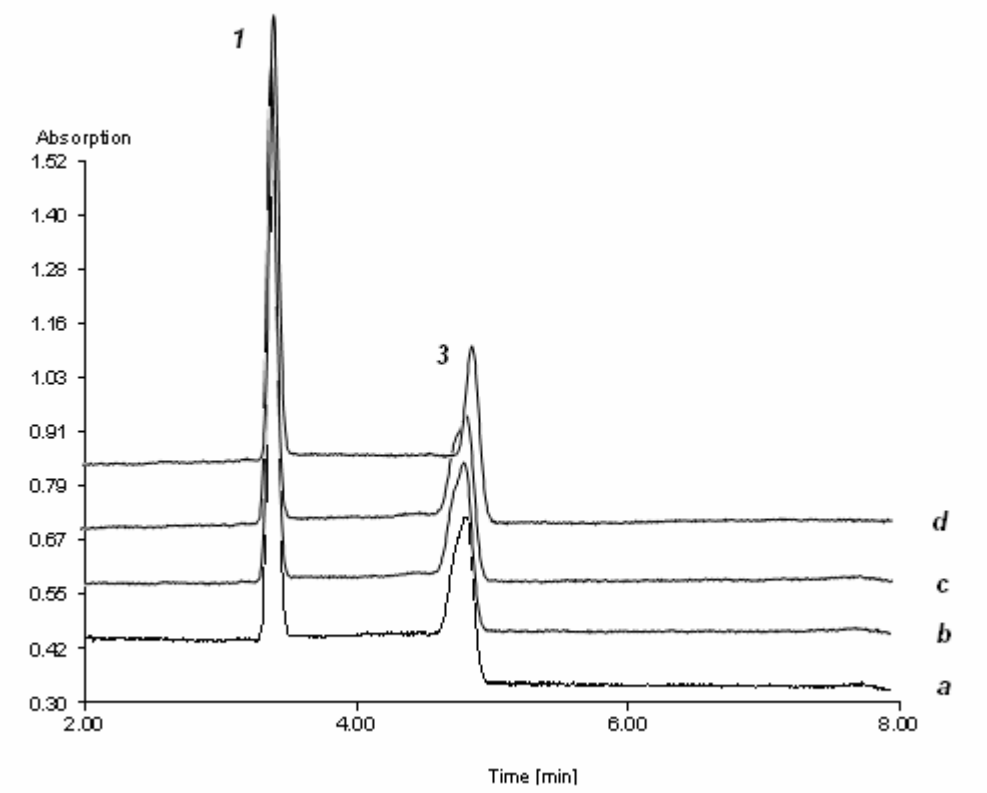


Fig. 23. The electropherogram of ß-casein analysis pH 4.0 with the presence of PEG 32 mg/mL using a bare fused-silica capillary. Peak 1: neostigmine bromide; peak 3: acetanilide. Acetate buffer pH 4.0 (50 mM), V = 18 kV, I ~ 46 µA, additional pressure: 100 mbar

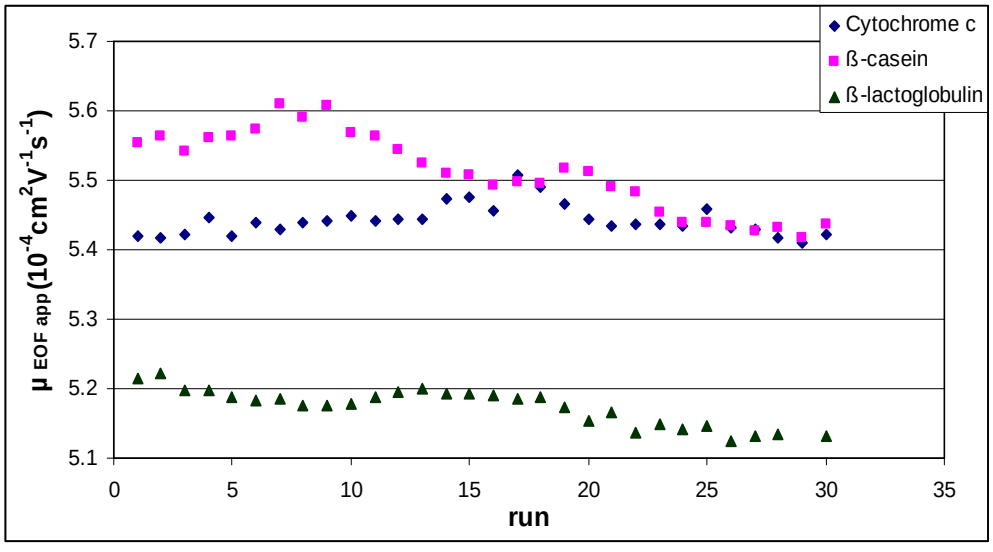


Fig. 24. Apparent EOF mobility of ß-lactoglobulin, cytochrome c and ß-casein analysis at pH 4.0 using a bare fused-silica capillary

Table 21. RSD (%) of apparent EOF mobility of ß-lactoglobulin analysis at different pH value

ß-lactoglobulin		RSD% $\mu_{EOF\ app}$	Number of run
pH 6.0			
without PEG	Control 1	0.722	30
	Protein	25.3	30
	Control 2	31.2	30
PEG 3.2 mg/mL	Control 1	5.22	30
	Protein	17.3	30
	Control 2	11.4	12
PEG 32 mg/mL	Control 1	1.17	30
	Protein	0.611	30
	Control 2	0.702	30
pH 5.0 (multyple use from pH 6.0)			
PEG 32 mg/mL	Control 1	0.488	30
	Protein	1.200	30
	Control 2	0.722	30
pH 4.0 (new capillary)			
PEG 32 mg/mL	Control 1	0.493	30
	Protein	0.517	30
	Control 2	0.998	30

Table 22. RSD (%) of apparent EOF mobility of cytochrome c analysis at pH 4.0

cytochrome c		RSD% $\mu_{EOF\ app}$	Number of run
pH 4.0			
PEG 32 mg/ml	Control 1	0.612	30
	Protein	0.410	30
	Control 2	0.532	30

Table 23. RSD (%) of apparent EOF mobility of ß-casein analysis at pH 4.0

ß-casein		RSD% μ_{EOFapp}	Number of run
pH 4.0			
PEG 32 mg/ml	Control 1	0.449	30
	Protein	1.05	30
	Control 2	0.374	30

3.1.4. Analysis of ß-lactoglobulin, cytochrome c and ß-casein using LPA-coated capillaries with HCl and phosphoric acid as rinsing agents

In order to solve the problem of protein adsorption many method developments using coated capillaries for electrophoresis are investigated. As discussed before, coated capillaries in CZE are preferable to uncoated ones to reduce the wall interactions of protein molecules. Yet

significant adsorption of proteins was also observed using coated capillaries [19, 45]. However, among the not perfect coating materials, linear polyacrylamide (LPA) was reported as the best to reduce adsorption and very stable at any conditions [30, 45, 46].

In order to maintain the quality of the capillary during protein separation, many investigations on rinsing procedures to remove adsorbed proteins in bare fused-silica capillaries have been reported, such as the rinsing with sodium hydroxide, sodium dodecyl sulphate and hydrochloric acid [59, 69, 70]. Rinsing either with NaOH or HCl is most routinely used, although a trace of proteins still remains on the capillary wall [59], whereas the rinsing with buffers containing SDS is likely 100% effective to remove adsorbed proteins. The desorption process of the protein is performed by formation adsorbed protein and SDS micelles [45, 59]. Nevertheless, this desorption process that are affected by driving electrophoretically SDS micelles is only effective within a short storage time which means only freshly adsorbed protein can be removed from capillary wall [45].

The effectiveness of high concentration acid (1 M HCl) was proven in the basic protein analysis using uncoated fused-silica capillaries. The fused-silica surface subsequently remains uncharged during analysis and acid pretreatment counteracts surface adsorption of proteins. As a result, a short rinsing of HCl offers a high reproducibility of EOF mobility and of migration times of proteins [69].

The rinsing with 2 M HCl was also effective for removing adsorbate from polyacrylamide-coated capillary. A weaker acidic solution (0.5 – 1.0 M) was not adequately efficient to remove proteins accumulated on the capillary wall. In 2 M HCl solution, proteins are strongly positively charged and possibly the non-coated surface of the capillary wall become uncharged. Thus, the electrostatic interaction between proteins and capillary wall is completely eliminated [46].

The EOF stability is often critical for the analysis of proteins by capillary electrophoresis, due to protein adsorption on the capillary wall. In order to overcome this problem, hydrochloric acid rinsing has been suggested by Mohabbati, et al. [46]. It was demonstrated that this procedure improved the repeatability of migration times and peak areas in short series. In order to investigate the general applicability of this approach, 60 runs of ß-lactoglobulin analysis were performed during three days. The capillary was regenerated using 2 M hydrochloric acid after the 30^{th} run, approximately 24 hours after the start of the series.

As the electroosmotic velocity is low compared to uncoated capillaries, additional pressure is applied, as described above, to perform analysis in a reasonable time-frame. The observed velocity depends on the EOF and the hydrodynamic flow-rate. Therefore, the apparent EOF is calculated from the measured migration times and is higher than the real occurring EOF. For practical reasons the apparent EOF will be discussed.

A RSD% of 4.94% for the apparent EOF mobility for all 60 runs was obtained. However, the RSD% values were much smaller within the first and the second 30 runs: 3.21% and 2.9%, respectively (Table 24). Thus, the regeneration using hydrochloric acid rinsing was not successful in this first experiment, probably due to ageing processes, which have been described before. After ageing proteins have been found, to be more difficult to remove from the capillary wall, especially if stored for more than 24 hours. Ageing is possibly caused by aggregation, unfolding, misfolding or generally denaturing of the adsorbed proteins [45]. Hints on such effects can be found in data given in Tables and Figures by Mohabbati, et al., although their work did not focus on this effect [46].

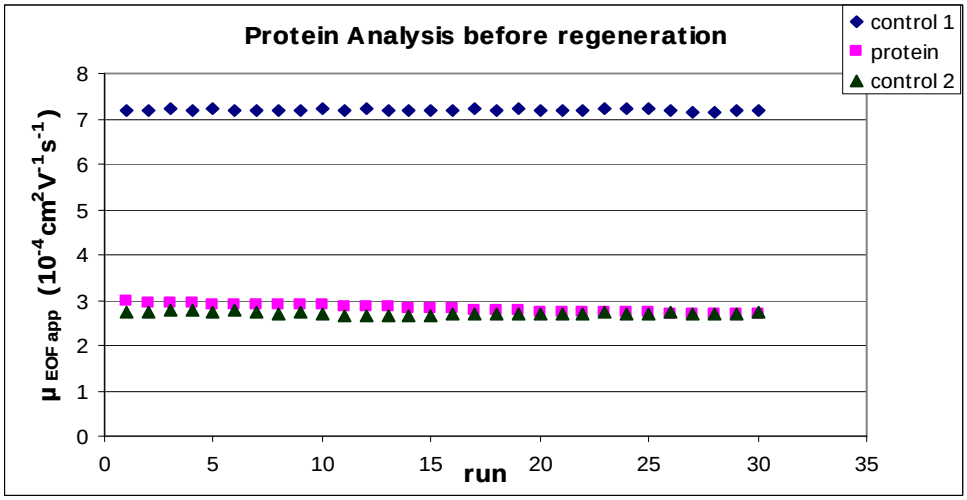


Fig. 25. Apparent EOF mobility of ß-lactoglobulin analysis at pH 5.5 before regeneration of the capillary using an LPA-coated capillary

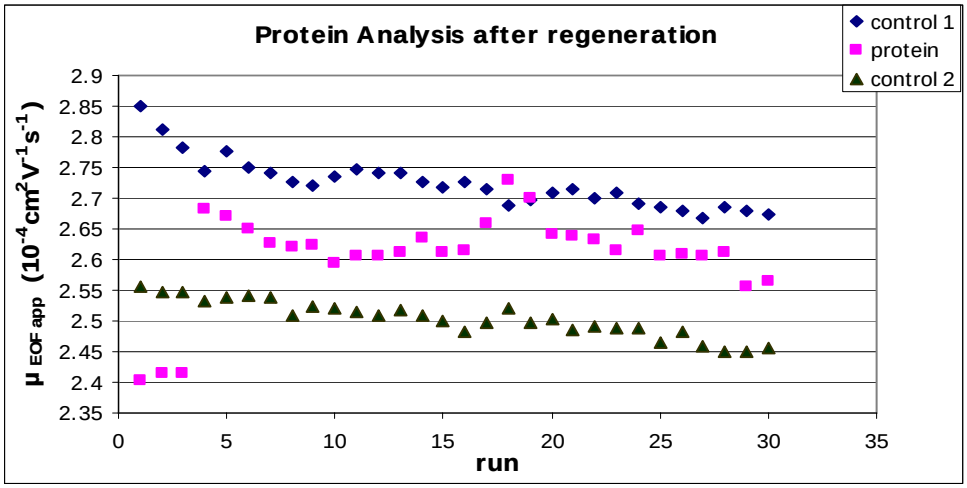


Fig. 26. Apparent EOF mobility of ß-lactoglobulin analysis at pH 5.5 after regeneration of capillary using an LPA-coated capillary

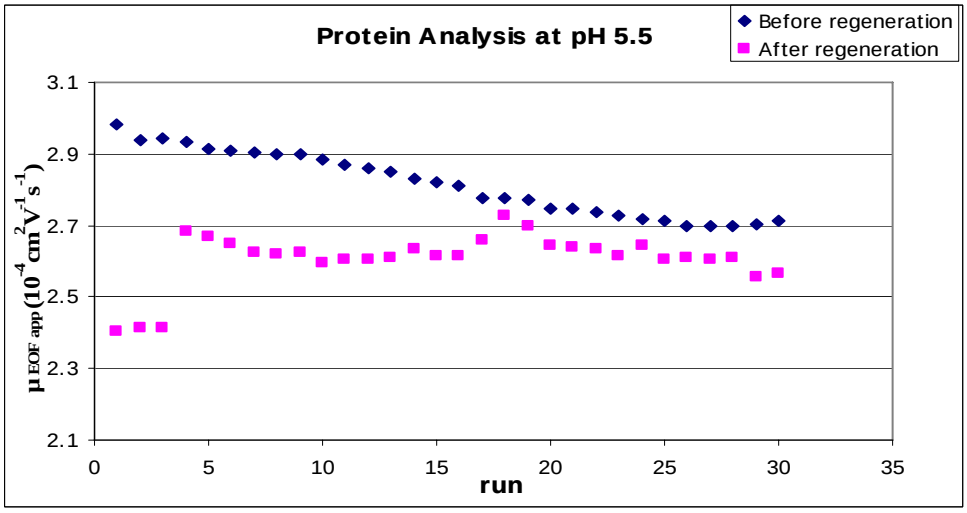


Fig. 27. The comparative apparent EOF mobility of ß-lactoglobulin analysis at pH 5.5 before and after regeneration of the capillary using an LPA-coated capillary

Table 24. RSD (%) of EOF mobility, migration time and peak area of proteins at pH 5.5 with an LPA coated capillary (Regeneration more than 24 hours)

pH 5.5		RSD% EOF marker			RSD% Internal Standard	
		t_{mig}	Peak Area	$\mu_{EOF\ app}$	t_{mig}	Peak Area
before regeneration	Control 1	0.283	1.402	0.283	0.401	1.52
	Protein (n=30)	3.19	13.4	3.206	2.52	1.028
	Control 2	1.088	1.924	1.092	0.474	1.032
after regeneration	Control 1	1.49	1.95	1.52	1.080	1.35
	Protein (n=30)	3.04	48.1	2.90	0.956	1.64
	Control 2	1.202	2.074	1.20	1.047	1.12
Total	Protein (n=60)	4.96	49.1	4.94	3.032	1.38

pH 5.5	RSD% Protein	
	t_{mig}	Peak Area
before regeneration	3.31	48.2
after regeneration	-	-

In order to improve the EOF repeatability, freshly adsorbed proteins should be removed before significant ageing takes places. Therefore, in the following experiments, the capillary was rinsed after every 10^{th} run for 5 minutes with 2 M hydrochloric acid, 5 minutes with water and 30 minutes with buffer, respectively. These conditions showed a very promising repeatability in short series. Therefore, the stability of the apparent EOF mobility was investigated using this rinsing regimen during long-term use, considering different types of proteins and different pH values, especially the ones close to the p*I* values of the proteins. Note that an apparent EOF mobility was determined and that this apparent parameter is given throughout the text. As an example, at pH 5.5 a real EOF of 3.3×10^{-5} $cm^2V^{-1}s^{-1}$ was obtained, which means approximately 10% of the EOF mobility found for bare fused-silica capillaries at neutral pH. A constant hydrodynamic flow has been applied in order to accelerate the method. This flow adds up to the EOF. However, as this flow was constant within each series, the results and their variability can well be compared between each other.

At first, ß-lactoglobulin was analyzed at pH 5.5 for 60 runs using a 2 M hydrochloric acid rinsing after every 10th run. As shown in Figure 28, the migration times of the internal standard and the EOF marker were highly reproducible, even though the buffer pH was close to the p*I* of ß-lactoglobulin. A change in the protein migration behavior itself was observed, but this is probably due to the long-term change in protein surface chemistry, e.g. hydrolysis or oxidation, which reduces the number of negative charges and therefore causes a higher mobility. However, an ageing effect was not found in this experiment, shown by the good repeatability of the apparent EOF mobility (RSD% = 2.88%, Table 25). Obviously 2 M hydrochloric acid was able to effectively remove adsorbed proteins on the linear polyacrylamide-coated capillaries. When an extraordinary high concentration of protein (175 µM) was analyzed, a decrease in the repeatability of the apparent EOF mobility was observed with RSD 5.27%. Similar to the analysis of 35 µM ß-lactoglobulin, a shift in the protein migration time was obviously observed (Figure 29). However, the repeatability was still by far better compared to results obtained without hydrochloric acid inter-rinses.

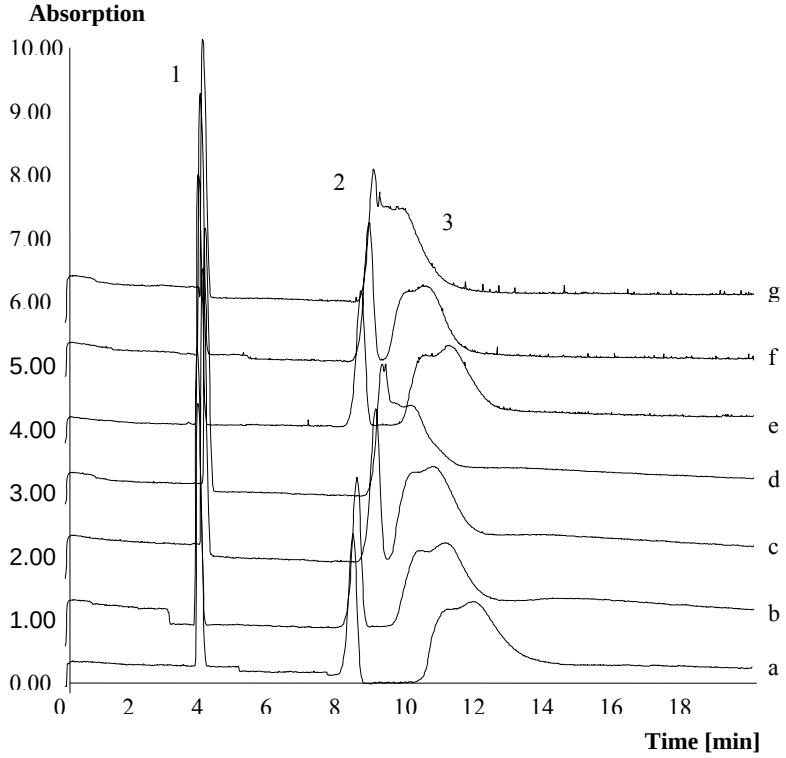


Fig. 28. Electropherograms of ß-lactoglobulin analysis (35 µM) at the a. 1st, b. 10th, c. 20th, d. 30th, e. 40th, f. 50th and g. 60th run at pH 5.5. Peak 1. neostigmine bromide (internal standard), 2. acetanilide (EOF marker), 3. ß-lactoglobulin. 50 mM acetate buffer, pH 5.5; 20 kV (60µA); additional pressure: 15 mbar; rinsing reagent: 2 M HCl

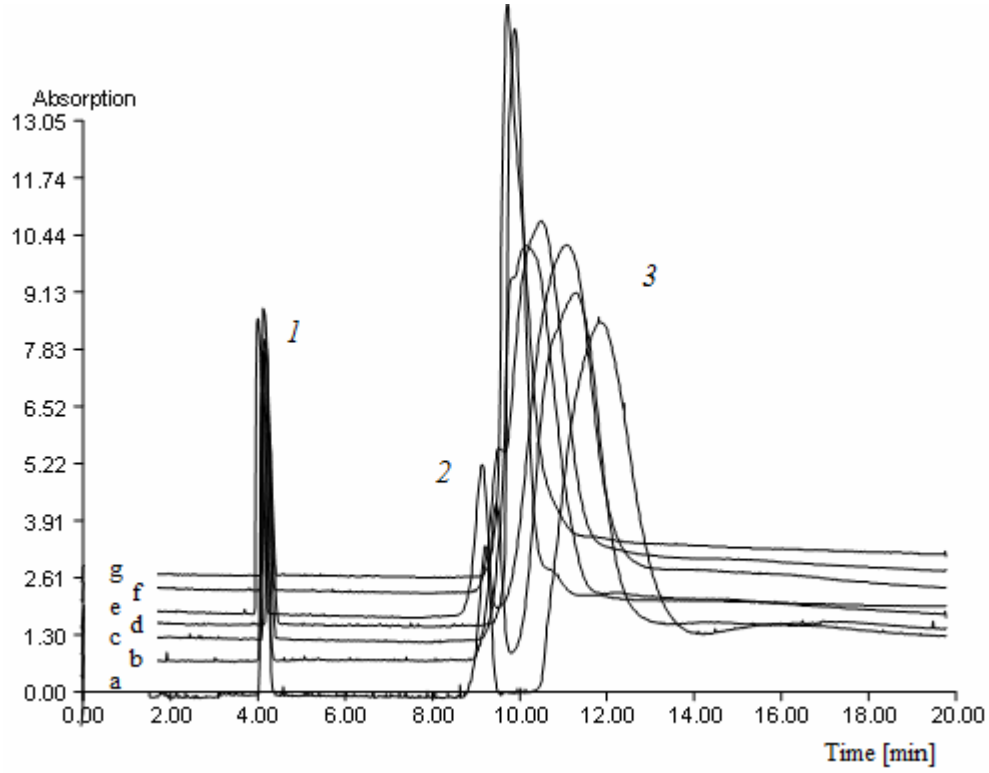


Fig. 29. Electropherograms of ß-lactoglobulin analysis (175 µM) at the a. 1st, b. 10th, c. 20th, d. 30th, e. 40th, f. 50th and g. 60th run at pH 5.5. Peak 1. neostigmine bromide (internal standard), 2. acetanilide (EOF marker), 3. ß-lactoglobulin. 50 mM acetate buffer, pH 5.5; 20 kV (60µA); additional pressure: 15 mbar; rinsing reagent: 2M HCl

The behavior of cytochrome *c*, ß-casein and a protein mixture containing both cytochrome *c* and ß-lactoglobulin was also investigated at pH 5.5. In the analysis of ß-casein, the interaction between ß-casein and the capillary wall still occurs during separation process. This interaction was facilitated by the less stability of ß-casein. It was shown by a decreasing peak area and then undetectable peak of ß-casein after several runs (Figure 30). In the other case, although cytochrome c is a basic protein, the reproducibility of its migration time and peak area is observed during the long-term measurement. It shows a high stability of cytochrome c, while the analysis was performed at pH 5.5 (Figure 31) and in the presence of ß-lactoglobulin in sample solution (Figure 32). Nevertheless, a remarkable stability of the apparent EOF mobilities of those analyses for 60 runs was obtained, values of RSD% < 3% were achieved. The results of those three respective analyses are shown in Table 1. The stability of apparent EOF mobilities indicated that HCl 2M is effective to remove adsorbate from capillary wall.

Table 25. Precision of protein migration time by linear polyacrylamide-coated capillaries at different pH values.

Protein	Concentration	pH	Rinsing Reagent	Number of runs	t_{mig} of EOF marker	$\mu_{EOF\ app}$	
					mean ± SD [min]	Mean ± SD [$10^{-4}cm^2V^{-1}s^{-1}$]	RSD%
ß-lactoglobulin	35 µM	5.5	2 M HCl	60	8.75 ± 0.254	2.74 ± 0.079	2.88
ß-lactoglobulin	175 µM	5.5	2 M HCl	60	9.82 ± 0.527	2.45 ± 0.129	5.27
ß-lactoglobulin	175 µM	5.5	3 M HCl	60	10.51 ± 0.372	2.29 ± 0.079	3.43
ß-lactoglobulin	175 µM	5.5	85 % (m/m) H_3PO_4	60	10.77 ± 0.263	2.23 ± 0.054	2.44
ß-casein	35 µM	5.5	2 M HCl	60	5.9 ± 0.173	4.07 ± 0.119	2.92
cytochrome c	35 µM	5.5	2 M HCl	60	10.6 ± 0.286	2.27 ± 0.06	2.67
ß-lactoglobulin + cytochrome c	each 35 µM	5.5	2 M HCl	60	8.97 ± 0.255	2.67 ± 0.077	2.87
ß-casein	35 µM	4.5	2 M HCl	30	10.5 ± 0.119	1.52 ± 0.017	1.15
cytochrome c	35 µM	4.5	2 M HCl	30	9.74 ± 0.221	1.64 ± 0.037	2.27
ß-casein	35 µM	3.5	2 M HCl	30	8.26 ± 0.42	2.91 ± 0.152	5.2
ß-casein	35 µM	3.5	85 % (m/m) H_3PO_4	30	8.28 ± 0.167	2.90 ± 0.06	2.08
cytochrome c	35 µM	3.5	2 M HCl	30	13.5 ± 0.285	1.19 ± 0.025	2.13

The apparent EOF mobility in this experiment showed a better repeatability compared to previous works using linear polyacrylamide-coated capillaries without hydrochloric acid rinsing inter-runs [45]. In the analyses using coated capillaries, an external pressure is applied to reduce the analysis time. Therefore, the repeatability was measured using the apparent EOF mobility. As the variability of the external pressure is very low, the RSD% of the EOF mobility must be higher than RSD% of the apparent EOF mobility.

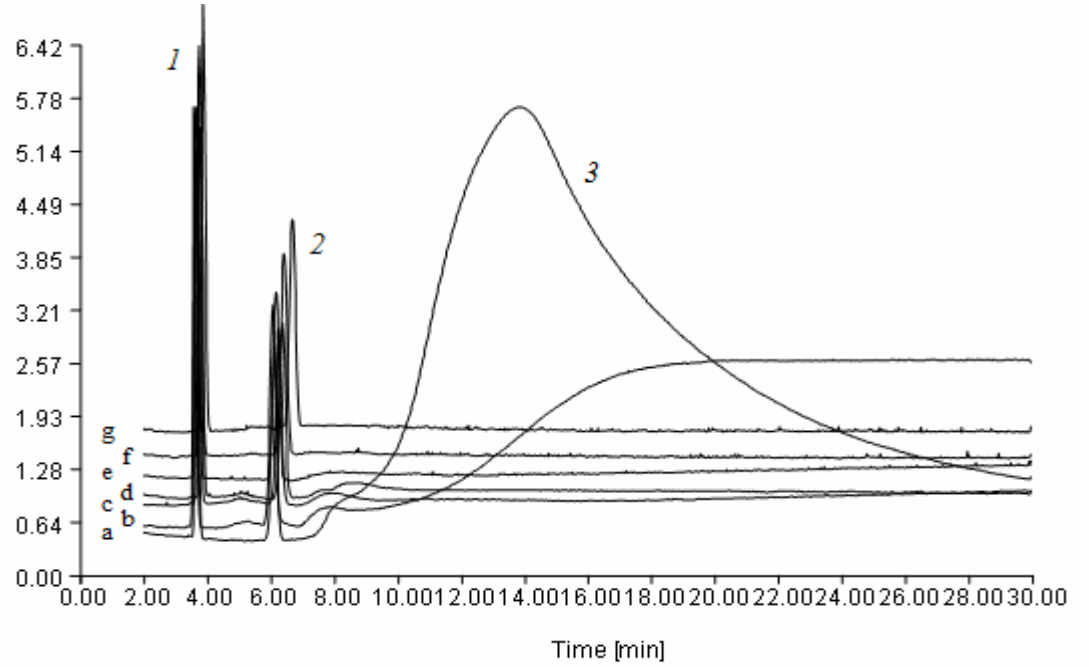


Fig. 30. Electropherograms of ß-casein analysis (35 µM) at the a. 1st, b. 10th, c. 20th, d. 30th, e. 40th, f. 50th and g. 60th run at pH 5.5. Peak 1. neostigmine bromide (internal standard), 2. acetanilide (EOF marker), 3. ß-casein. 50 mM acetate buffer, pH 5.5; 20 kV (60 µA); additional pressure: 25 mbar; rinsing reagent: 2 M HCl

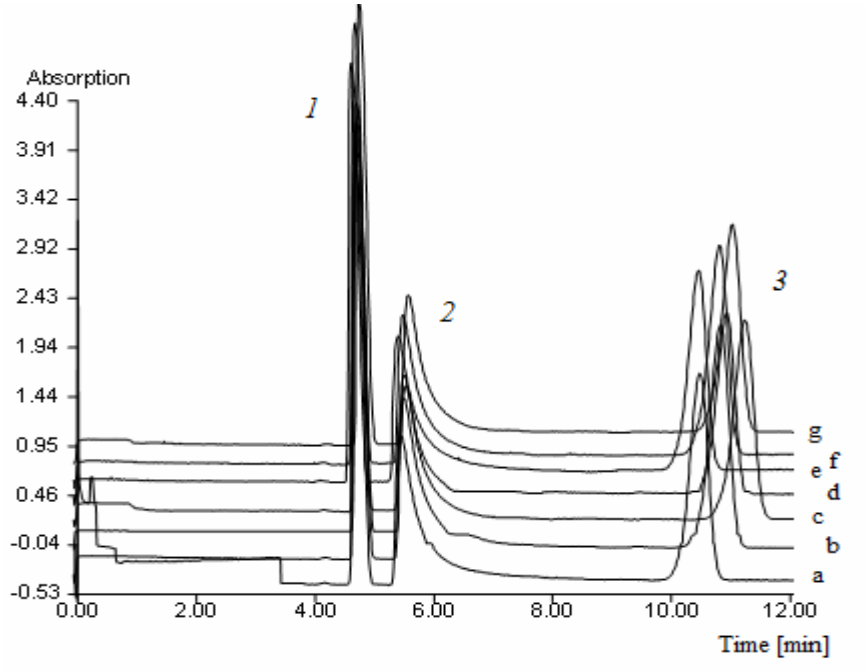


Fig. 31. Electropherograms of cytochrome c analysis (35 µM) at the a. 1st, b. 10th, c. 20th, d. 30th, e. 40th, f. 50th and g. 60th run at pH 5.5. Peak 1. neostigmine bromide (internal standard), 2. cytochrome c 3. acetanilide (EOF marker). 50 mM acetate buffer, pH 5.5; 20 kV (60µA); additional pressure: 15 mbar; rinsing reagent: 2M HCl

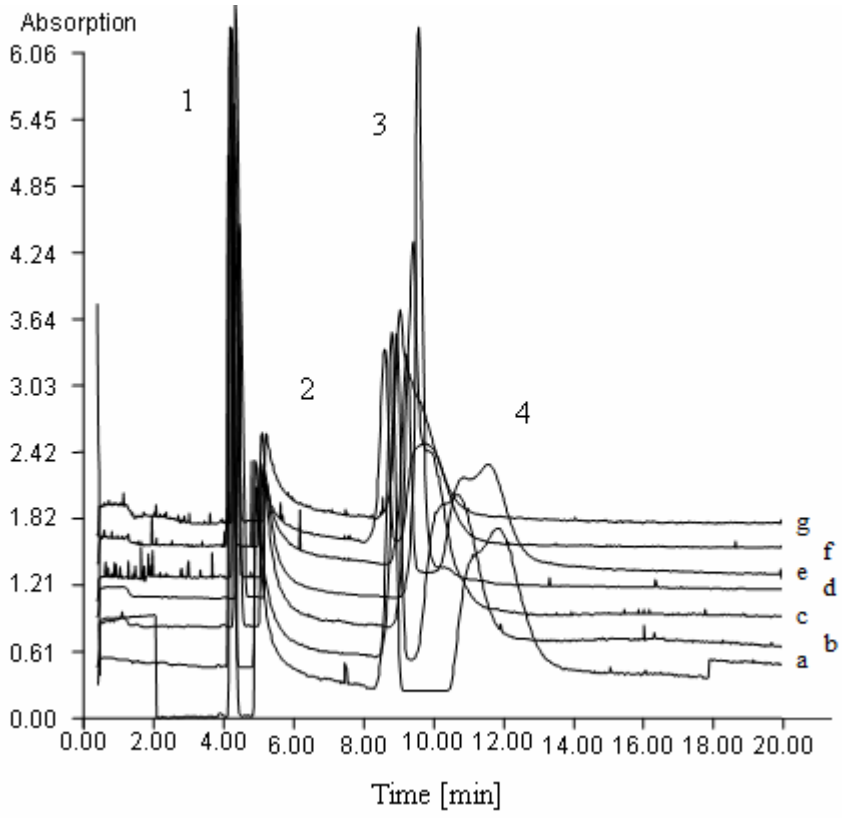


Fig. 32. Electropherograms of cytochrome c and ß-lactoglobulin analysis (each 35 µM) at the a. 1st, b. 10th, c. 20th, d. 30th, e. 40th, f. 50th and g. 60th run at pH 5.5. Peak 1. neostigmine bromide (internal standard), 2. cytochrome c, 3. acetanilide (EOF marker), 4. ß-lactoglobulin. 50 mM acetate buffer, pH 5.5; 20 kV (60µA); additional pressure: 15 mbar; rinsing reagent: 2M HCl

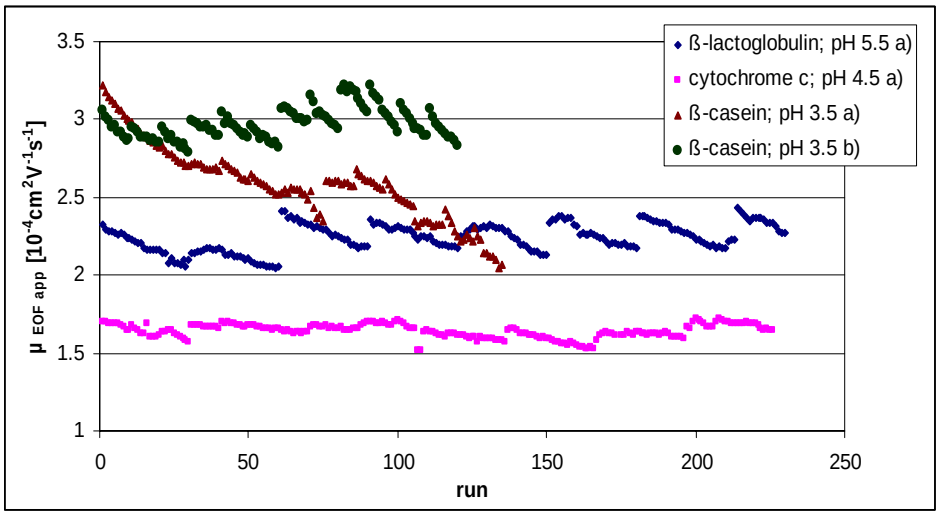


Fig. 33. Long-term behavior of the apparent EOF mobility for various proteins and buffer pH values; compare Table 26. Rinsing reagents: a) 2 M hydrochloric acid; b) 85% (m/m) phosphoric acid

Table 26. Precision of protein analysis (35 µM concentrations each) during long-term use using linear polyacrylamide-coated capillaries at different pH values

Protein	pH	Rinsing Reagent	Number of runs	t_{mig} of EOF marker	$\mu_{EOF\ app}$	
				mean ± SD [min]	mean ± SD [$10^{-4}cm^2V^{-1}s^{-1}$]	RSD%
ß-lactoglobulin	5.5	2 M HCl	230	10.7 ± 0.43	2.24 ± 0.089	3.96
cytochrome c	4.5	2 M HCl	226	9.76 ± 0.264	1.64 ± 0.044	2.67
ß-casein	3.5	2 M HCl	135	9.37 ± 0.89	2.59 ± 0.244	9.42
ß-casein	3.5	85 % (m/m) H_3PO_4	120	8.084 ± 0.262	2.97 ± 0.098	3.31

The influence of ß-casein and cytochrome *c* on the apparent EOF mobility was also investigated using an acetate buffer at pH 4.5 and a formate buffer at 3.5. As shown in the Table 25, the repeatability of the apparent EOF mobility was good for both protein analyses at pH 4.5 and cytochrome *c* analysis at pH 3.5 with RSD% < 3%. Only ß-casein at pH 3.5 showed an impaired precision (RSD% = 5.2%). ß-casein is known to easily undergo conformational changes causing a flat structure with multiple binding sites to surfaces. These changes facilitate a strong and often irreversible adsorption [71].

The effectiveness of rinsing with 2 M hydrochloric acid after every 10^{th} run for removing adsorbed proteins was also evaluated for a long-term use at various pH values. Figure 33 shows apparent EOF mobilities for 230 runs of ß-lactoglobulin analysis at pH 5.5, for 226 runs of cytochrome *c* analysis at pH 4.5 and for 135 runs of ß-casein at pH 3.5; one run took approximately 20 min. In this case, the apparent EOF mobility remains quite stable at pH 5.5 and 4.5 with RSD% 3.96% and 2.67%, respectively. Nevertheless, unstable apparent EOF mobility was observed for the analysis of ß-casein at pH 3.5 with an RSD% of 9.42% (as shown in Table 26).

2 M hydrochloric acid is successfully applied in general, except for two series of ß-casein analysis at low pH and high concentration of ß-lactoglobulin, in which inferior repeatability was obtained. In order to improve the repeatability of the apparent EOF mobility in both series, other rinsing reagents such as 3 M hydrochloric acid and 85% (m/m) phosphoric acid were applied. As shown in Table 25, the use of 3 M hydrochloric acid compared to 2 M hydrochloric acid offered better repeatability of the apparent EOF mobility for the analysis of highly concentrated ß-lactoglobulin at pH 5.5, RSD% being 3.43% (n=60). In this case, the

use of 85% (m/m) phosphoric acid still offered even better precision with RSD% 2.44%. The viscosity of phosphoric acid 85% (m/m) is significantly higher compared to water, but this did not cause any difficulty in our experiments. The effectiveness of the rinsing with 85% (m/m) phosphoric acid was also demonstrated for the analysis of ß-casein at pH 3.5 for 120 runs (RSD% 3.31%, Table 26). Because of the high effectiveness of 85% (m/m) phosphoric acid as rinsing reagent, higher concentrations (e.g. 5 M) of hydrochloric acid were not further tested, also to avoid hazard for the CE instrument from gaseous hydrochloric acid.

3.2. Capillary isoelectric focusing

Capillary isoelectric focusing is a high-resolution technique used for protein separation. However, in order to make this technique acceptable for routine analysis, improving reproducibility is still a major issue. Highly-concentrated proteins frequently cause protein adsorption at their isoelectric point. This is observed as well using CIEF. Consequently, irreproducibilities of migration time and peak area are continuously found. Furthermore, capillaries may be blocked even in short measurement series. Up to now, precision has been only reported for short measurement series [26, 40, 51, 72, 73]. In order to improve the reproducibility in a long term analysis, many methods have been developed and investigated.

3.2.1. Cleaning LPA-capillary surfaces

Capillary-surface quality significantly influences the performance of protein separation in CIEF. Protein adsorption occurs on the capillary surface, changing the EOF and the movement of the analytes. Consequently, low precision of protein analysis resulted. In order to keep the good quality of the capillary surface, a rinsing procedure after protein separation was introduced. Many investigations on rinsing procedures have been reported for the analysis of proteins using CZE. Among these, alkaline rinsing solutions or solutions containing SDS, which are very useful in CZE, are not suitable for CIEF, because they may damage or irreversibly change the surface properties of the usually employed polyacrylamide-coated capillaries [45, 51]. In previous experiments, the use of hydrochloric acid was also successfully applied on the polyacrylamide-coated capillaries to improve the reproducibility of migration times and peak areas in protein analysis. Another rinsing reagent, namely phosphoric acid 85% m/m was also effective to be applied especially for highly concentrated protein samples or for the more easily defolding ones. Several rinsing and storage procedures

for coated capillaries such as water rinsing, water rinsing followed with N_2 rinsing before capillary storage, or a storage of the capillary in distilled water were also described [40, 46]. As reported in this work, new rinsing procedures after protein separation and during capillary storage are designed to improve the precision of protein separations by isoelectric focusing.

In order to perform preliminary experiments by CIEF, varieties of hydrochloric acid concentrations and phosphoric acid 85% (m/m) were tested in myoglobin analysis by isoelectric focusing (Figure 34). Hydrochloric acid was efficient to remove adsorbed proteins from the capillary wall. Meanwhile, phosphoric acid 85% (m/m) was not successful for removing adsorbed protein from capillary wall. It was confirmed by capillary blockage that still occurred, even though capillary was rinsed by phosphoric acid 85% (m/m). Highly-concentrated proteins at their isoelectric point during focusing step caused adsorbed proteins focused on one point of capillary. If adsorbed protein cannot be removed from capillary, capillary blockage occurred. In a concentration range of 2M to 6M, 3M of hydrochloric acid is stronger than 2M for removing adsorbed proteins; meanwhile 6M of hydrochloric acid can give hazard for CE instrument from gaseous hydrochloric acid. The optimal results were achieved by 3M concentration that was considered by RSD% calculation of migration time and peak area of myoglobin during consecutive runs (Figure 35). Henceforth, myoglobin, ß-lactoglobulin and ovalbumin as model proteins and 3M hydrochloric acid as a rinsing reagent were investigated by isoelectric focusing (Figure 36).

Firstly, proteins were analyzed in 27 runs within 4 days (first day from 1^{st} to 5^{th} run, second day from 6^{th} to 11^{th} run, third day from 12^{th} to 19^{th} run and fourth day from 20^{th} to 27^{th} run). A capillary rinse with 3M hydrochloric acid has been performed after each run for 5 minutes, followed with water for 20 minutes. At the end of each analysis day, the capillary was rinsed with water and afterwards both capillary ends were immersed into water vials. These rinsing and storage procedures can be applied in long-series without any capillary blockage. However, the precision of migration time and peak area has not yet shown completely satisfying. Results of RSD% are in a range of 26-35% and 52-62% (n=27), respectively. As shown in Figure 37, significant changes of migration time and peak area were observed from day to day.

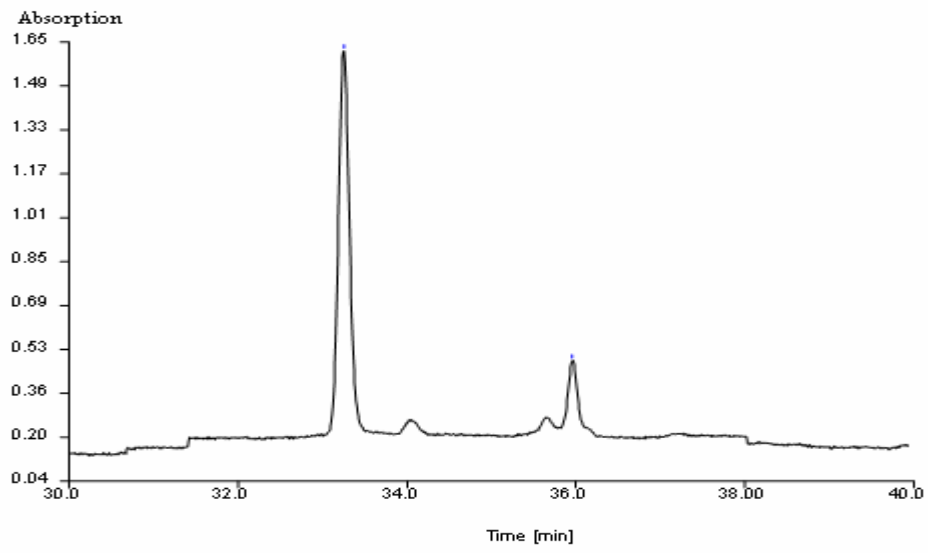


Fig. 34. Separation of myoglobin and side compounds by CIEF

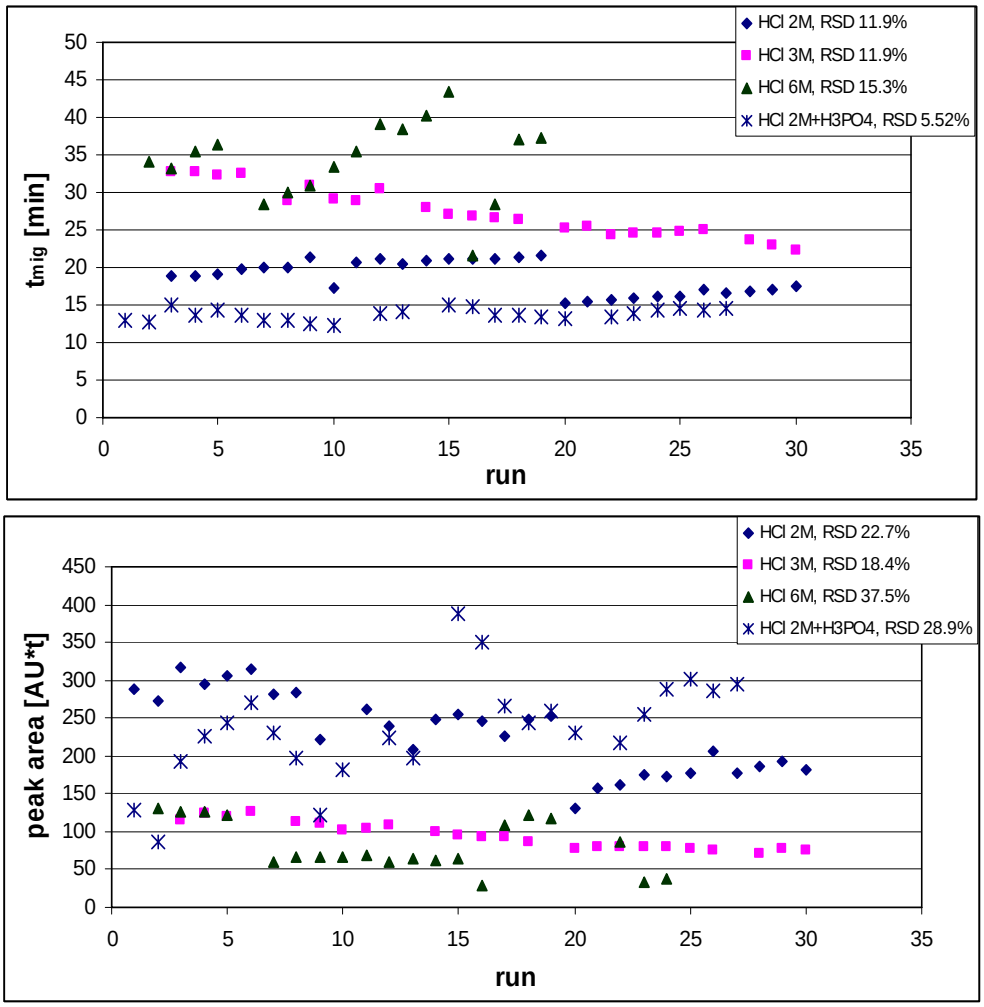


Fig. 35. Precision of protein analysis: migration time and peak area with the difference of rinsing reagents.

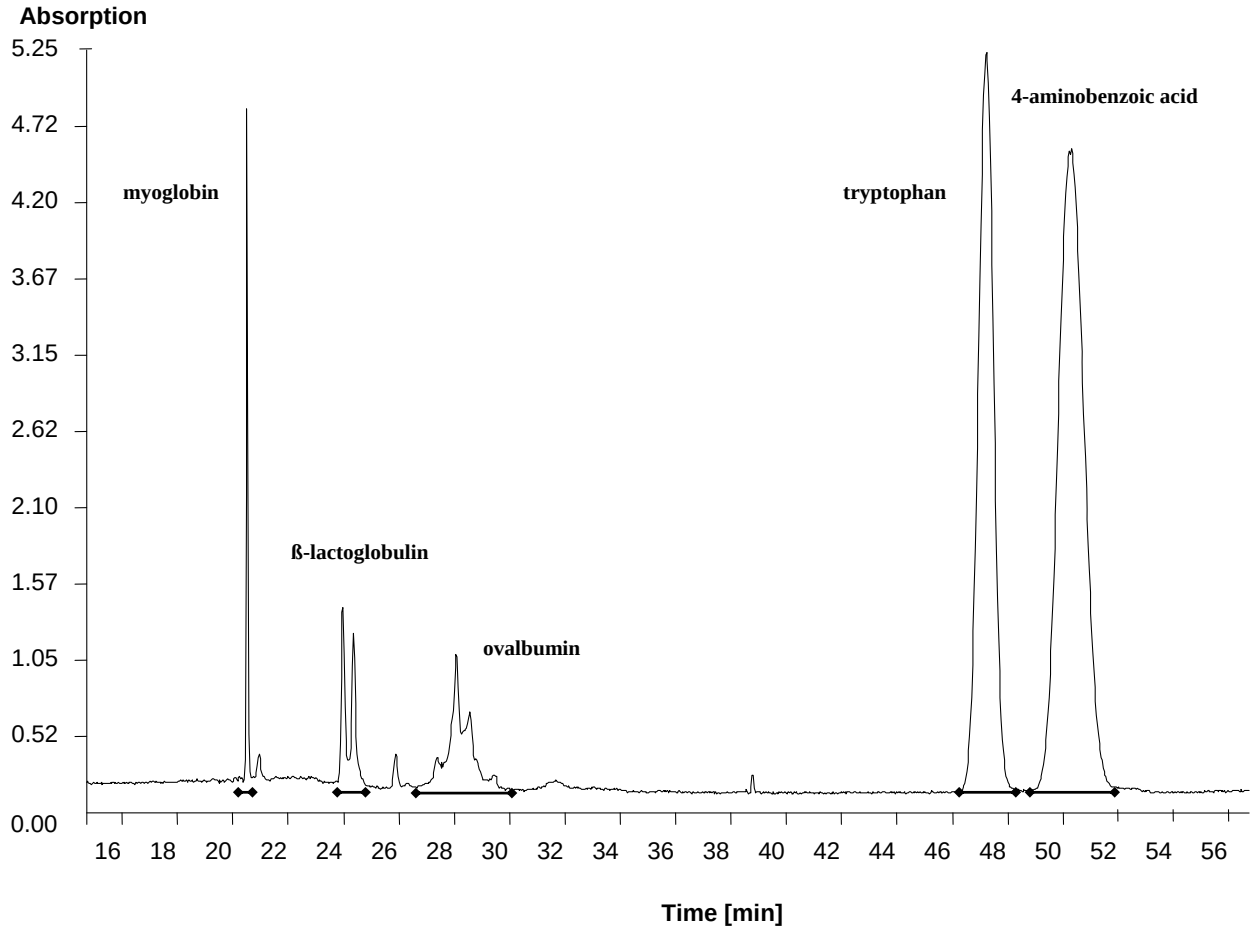


Fig. 36. Separation of proteins and their related compounds: myoglobin (0.3 mg/mL; p*I*: 6.8-7.4 [45, 51]; t_{mig}: 20-22 min), ß-lactoglobulin (0.6 mg/mL; p*I*: 4.83-5.4 [45, 51]; t_{mig}: 23.5-26 min), ovalbumin (1.2 mg/mL; p*I*: 5.1 [45, 51]; t_{mig}: 26-32 min) and internal standards: tryptophan (0.1 mg/mL; p*I* 5.9) and 4-aminobenzoic acid (0.02 mg/mL; p*I* 3.9) using a LPA-coated capillary. Carrier ampholyte solution: 2% Pharmalyte 3-10 in 0.8% HPMC solution; anolyte: 10 mM H_3PO_4; catholyte: 20 mM NaOH; T: 23°C. Using PrinCE 550 CE system, single-step CIEF was performed at a voltage of 30 kV (0.3 – 4.5 µA; refer to section 3.2.2.2.2). Integration has been performed in the marked boundaries.

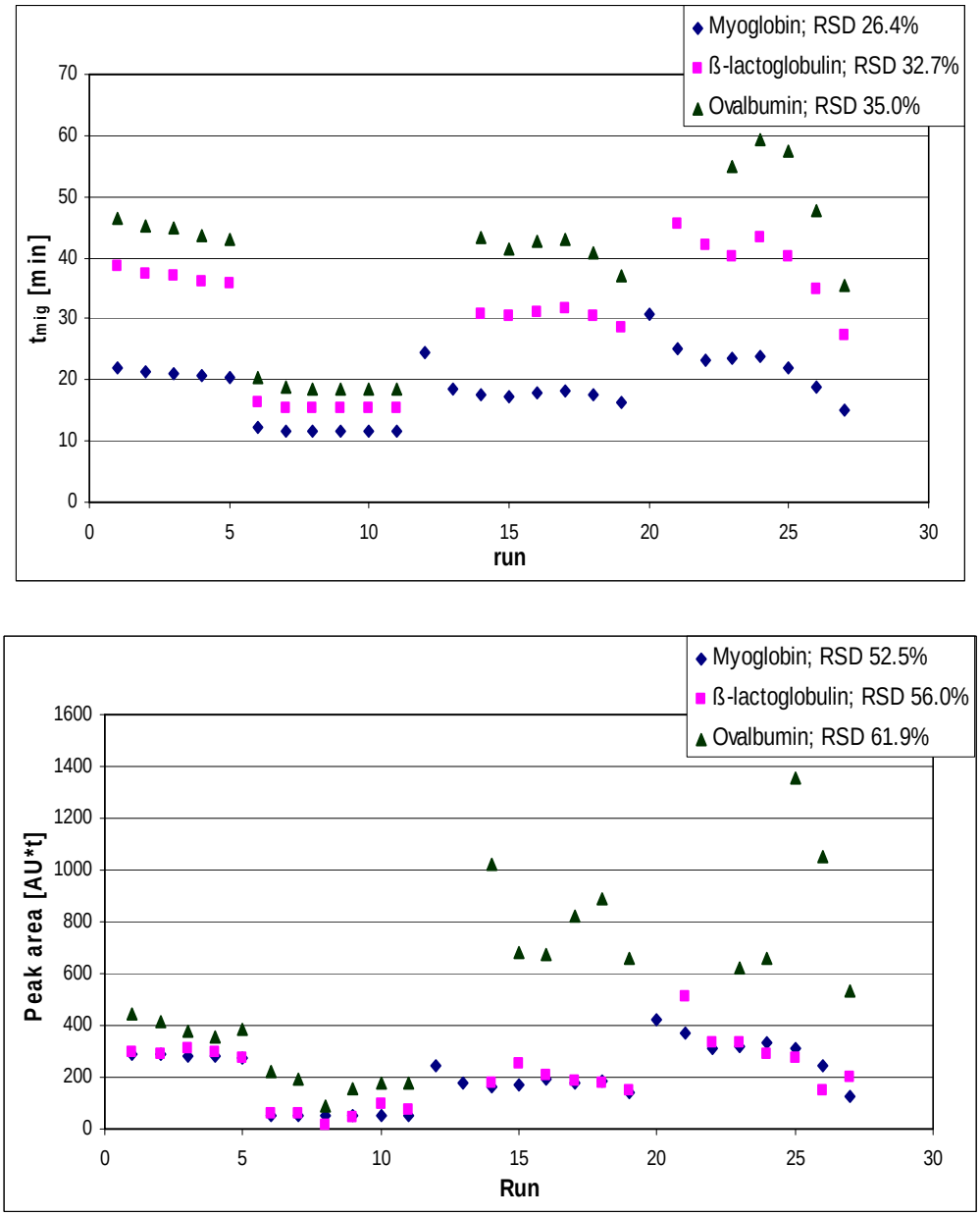


Fig. 37. Precision of protein analysis: migration time and peak area. The capillary was kept immersed in water in stand still. Using the UniCAM Crystal 310 CE System, the focusing step was performed at a voltage of 30 kV (0.9 – 8.4 µA) for 10 minutes and continued with a mobilization step using a pressure of 30 mbar. Integration has been performed in the boundaries given in Figure 36. Reported migration times refer to the highest peaks.

3.2.2. Investigation of further error sources

3.2.2.1. The fluctuation of room temperature

The precision of protein analysis with isoelectric focusing possibly depends on temperature control during the separation process. The electrophoretic and electroosmotic mobility increase with increasing temperature. Thus, the major effect of increasing temperature is to shorten the analysis time in the presence of electroosmotic flow. Even though constant temperature has been programmed, temperature homogeneity of the whole capillary cannot be reached easily. Instrument design does not allow for complete thermostatting. This is true for all existing CE instruments [74].

In our investigation, the temperature of 20°C and 25°C was programmed in the 1st – 16th runs and 17th – 32nd runs, respectively. Usually, the room temperature was in the same range. During the series of protein analysis, the set temperature was maximally exceeded by 7°C. However, an influence on migration time and peak area was never noted. Therefore, the effects of temperature are only minor (Figure 39).

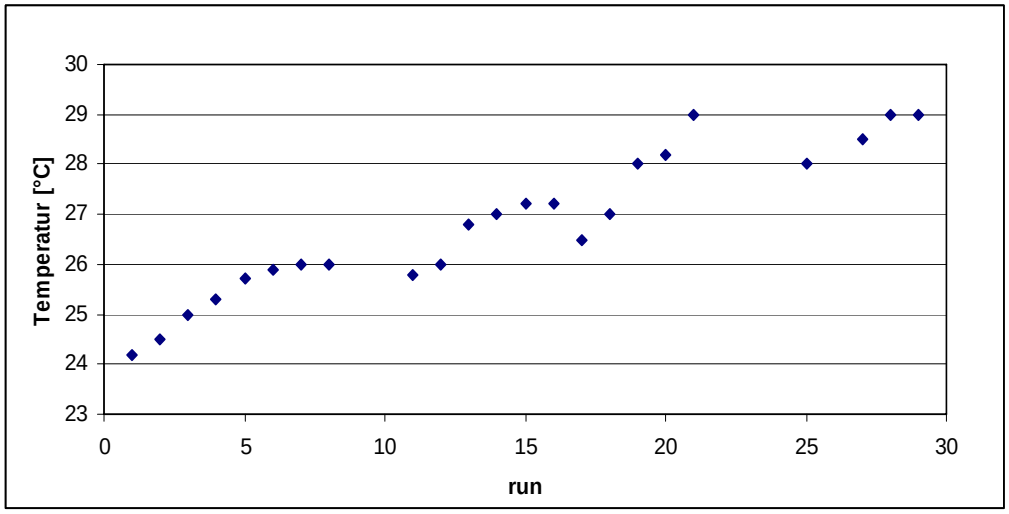


Fig. 38. Room temperature

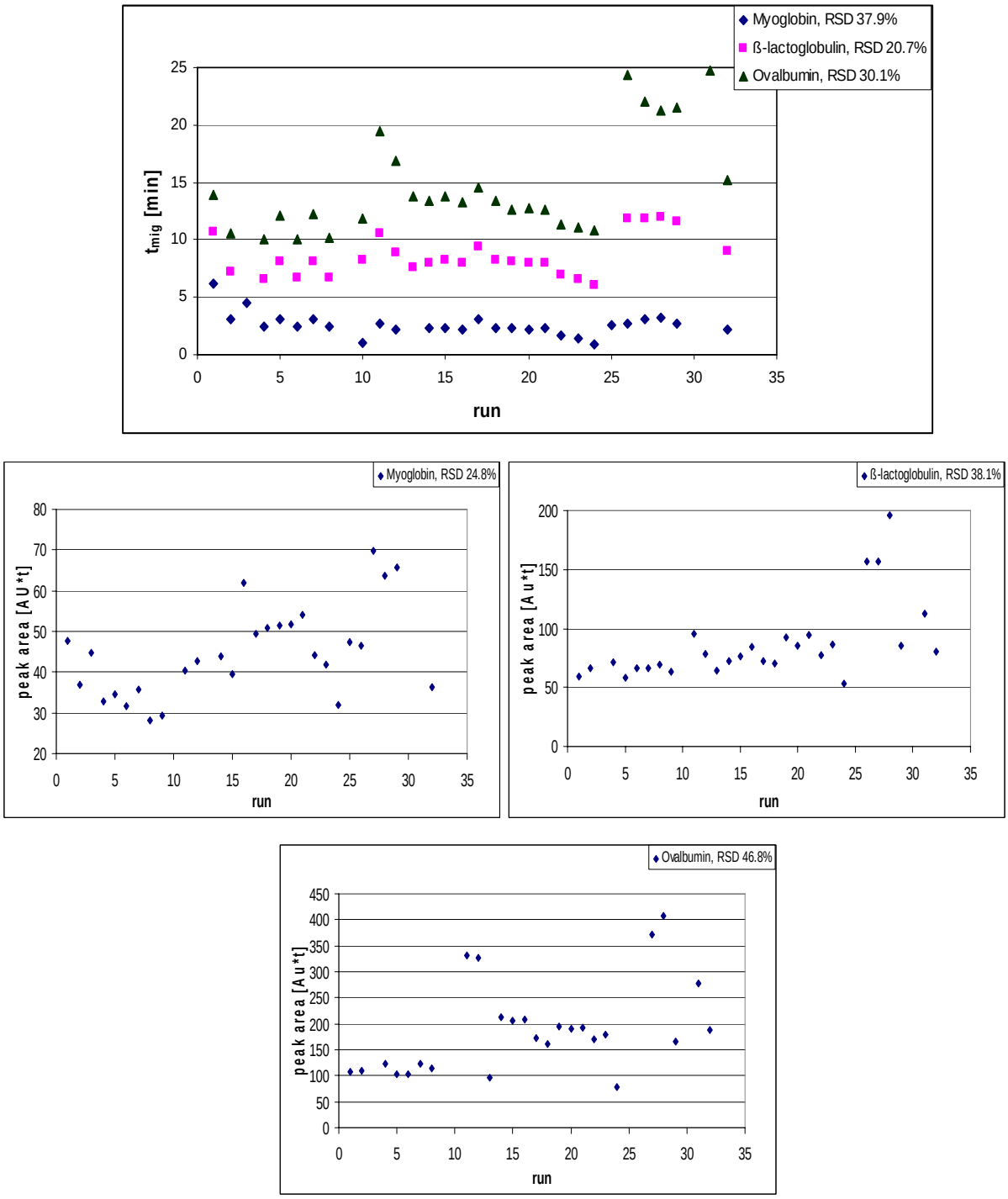


Fig. 39. Precision of protein analysis: migration time and peak area. Using the UniCAM Crystal 310 CE System, the focusing step was performed at a voltage of 30 kV (0.9 – 8.4 µA) for 10 minutes and continued with a mobilization step using a pressure of 30 mbar. Temperature control was programmed 20°C (1st – 16th runs) and 25°C (17th – 32nd runs). Integration has been performed in the boundaries given in Figure 36.

3.2.2.2. Alteration of surface structure

A change of surface structures on coated capillaries is able to influence the precision on protein analysis strongly. Therefore, an alteration of the surface structure in our investigation was assumed as a cause for the day-to-day irreproducibility of migration time and peak area. The long-term capillary storage at the end of an analysis day could cause e.g. bulged structures on the coated capillary, as observed in previous works [9]. Consequently, the EOF can be strongly influenced during a series of measurements. In principle, these assumed surface changes could be prevented by the following two strategies.

3.2.2.2.1. Capillary maintenance

First, if capillary storage is needed during routine analysis, water rinsing could be done by applying additional pressure and high voltage when the capillary was not used. This method would prevent the formation of crystals that could be produced when the filling of the capillary was not moving. A voltage 30 kV and a pressure 300 or 700 mbar were used in our investigation. No difference of reproducibility was observed when pressure 300 or 700 mbar was applied.

As shown in Figure 40, this rinsing procedure during capillary storage offered a precision improvement of migration time and peak area with RSD% in a range of 14-16% and 15-27% (n=59), respectively.

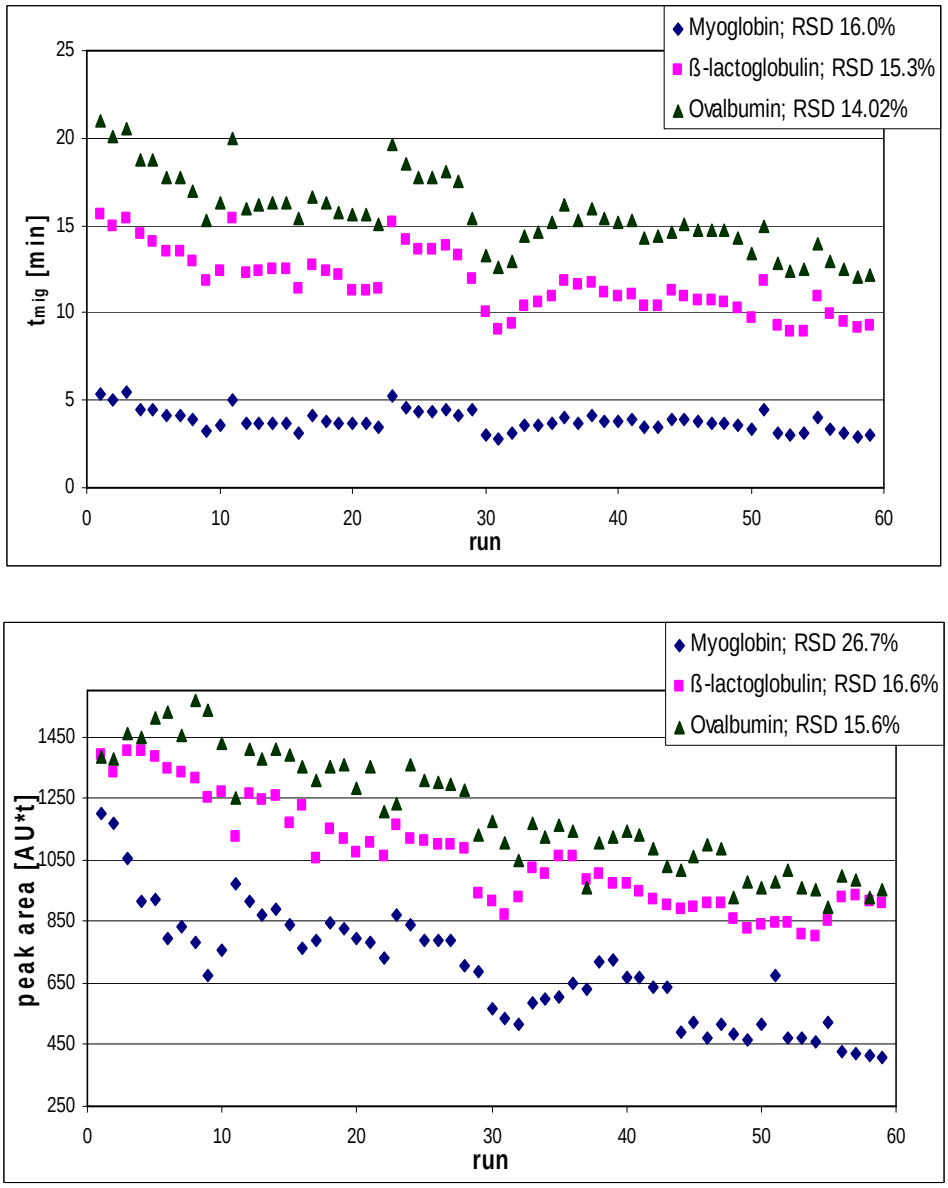


Fig. 40. Precision of protein analysis: migration time and peak area. During the series, a complete stand still was avoided by continuous water rinsing by applying pressure and high voltage. Using the UniCAM Crystal 310 CE System, the focusing step was performed at a voltage of 30 kV (0.9 – 8.4 µA) for 10 minutes and continued with a mobilization step using a pressure of 30 mbar. Integration has been performed in the boundaries given in Figure 36.

3.2.2.2.2. Consecutive runs

The second way to prevent the build-up of crystallization is by performing runs consecutively. It was performed without interruption during analysis runs. Therefore, the chance for build-up of crystallization on the capillary wall should be reduced.

The instrument used in earlier analysis has a single lift in which the outlet vials are not automatically changeable. The outlet vial contains sodium hydroxide as catholyte. However, the rinsing solution also reaches the outlet vial during the rinsing program. It will cause the change of catholyte pH values and then change of the pH gradient on the capillary. A switch to another instrument (PrinCE 550 CE System) was performed to facilitate consecutive runs. This PrinCE Instrument provides a double lift system permitting the outlet vial automatically to be changed according to the program. The running program could be set for a whole day and empty vials could be used as outlet in the period of capillary rinsing. Therefore, rinsing solutions such as hydrochloric acid, water, and sodium chloride reach the empty vial and contaminated sodium hydroxide in outlet vials could be prevented.

Earlier, protein separations using the UniCAM Instrument were performed with focusing steps for 10 minutes, continued with a mobilization step applying a pressure of 30 mbar. The effective capillary length in the PrinCE Instrument was shorter than in the UniCAM Instrument. As a consequence, the analysis time dropped. Therefore, additional pressure to mobilize the analytes was not needed using the PrinCE Instrument. Proteins were focused while they were transported towards the detector by EOF. In this case, single-step CIEF was performed while the coated capillary was used.

The effectiveness of consecutive runs to avoid assumed crystallization processes during capillary storage was also confirmed for the protein analysis. The electropherogram is shown in Figure 36. The precision of protein analysis was further improved with RSD% values less than 10% in long-term measurements (Figure 41). The calculated peak area reproducibility was referred to runs from 45 to 130.

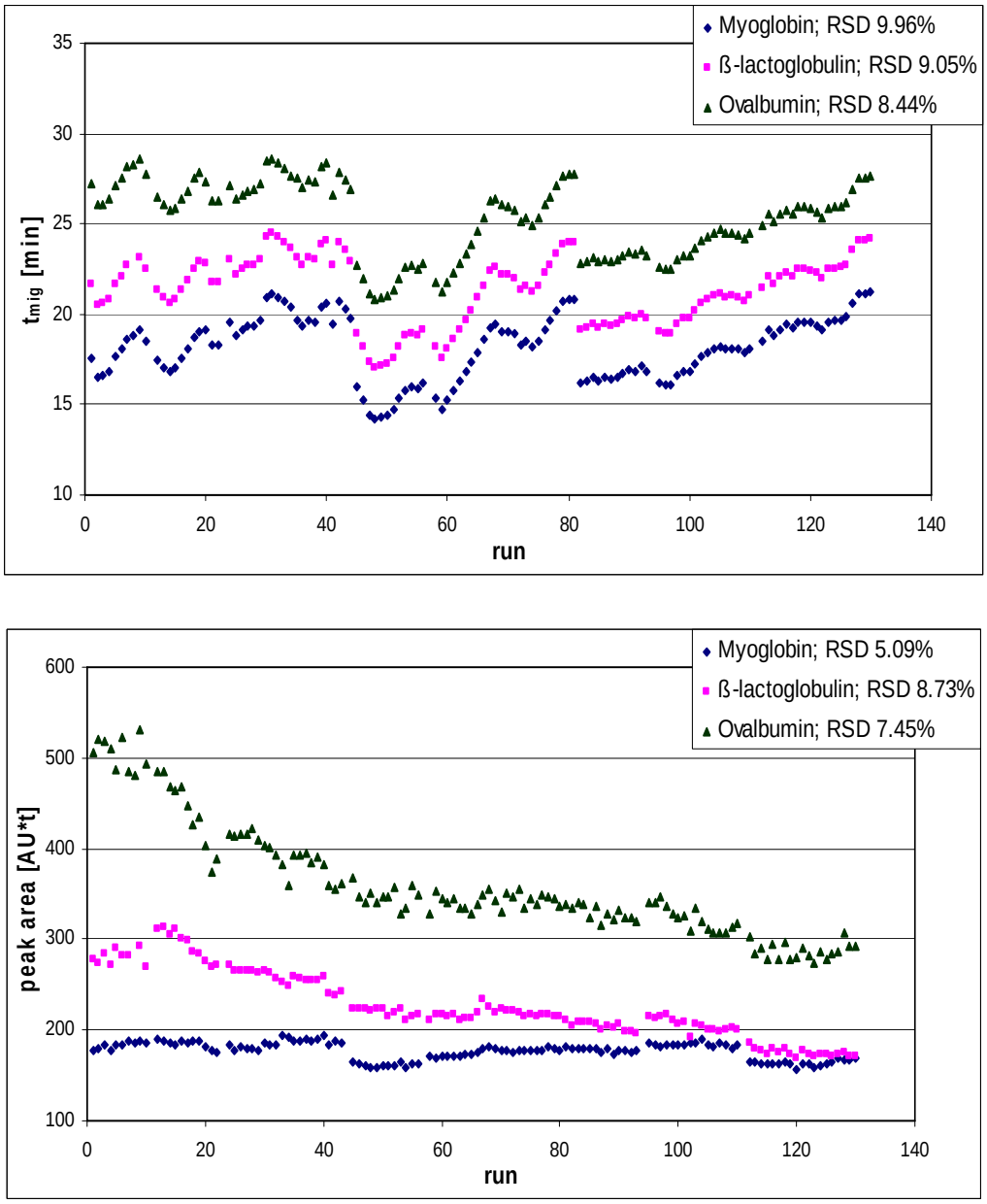


Fig. 41. Precision of protein analysis: migration time (different scale compared to Figure 37 and 40) and peak area (calculated precision refer to runs from 45 to 130). This series was performed continuously without interruptions during analysis runs. Separation conditions were the same as in Figure 36 using PrinCE 550 CE System. Single-step CIEF was performed at a voltage of 30 kV (0.3 – 4.5 µA). Integration has been performed in the boundaries given in Figure 36.

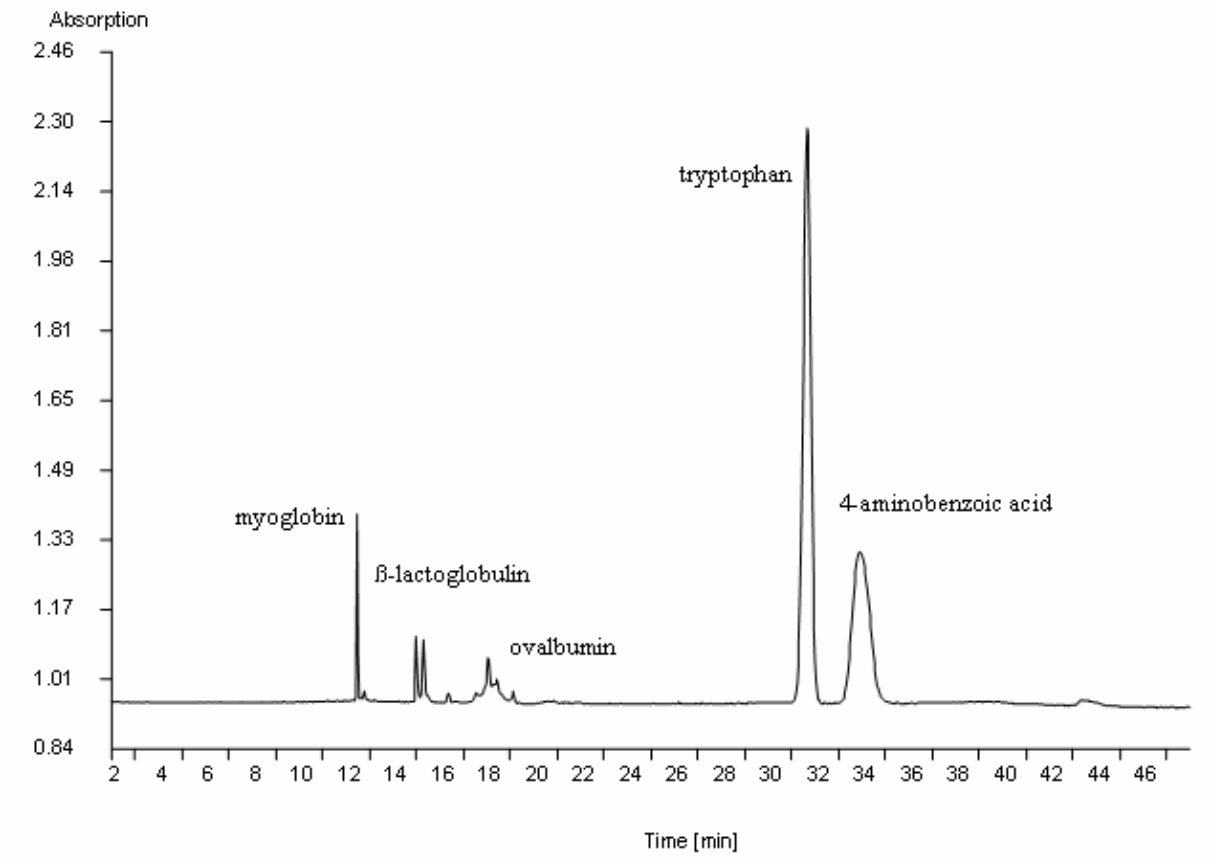


Fig. 42. Separation of proteins and their related compounds: myoglobin (0.3 mg/mL; p*I*: 6.8-7.4 [45, 51]), ß-lactoglobulin (0.6 mg/mL; p*I*: 4.83-5.4 [45, 51]), ovalbumin (1.2 mg/mL; p*I*: 5.1 [45, 51]) and internal standards: tryptophan (0.1 mg/mL; p*I* 5.9) and 4-aminobenzoic acid (0.02 mg/mL; p*I* 3.9). Carrier ampholyte solution: 2% Pharmalyte 3-10 in 0.8% HPMC solution; anolyte: 10 mM H_3PO_4; catholyte: 20 mM NaOH; T: 23°C. Using PrinCE 550 CE system, the focusing and mobilization step was performed in the same time at a voltage of 30 kV and additional pressure 25 mbar (0.3 – 4.5 µA; refer to section 3.2.2.2.2).

Decreasing the focusing time was also evaluated in this experiment to improve the reproducibility in protein analysis. During the focusing step, proteins become highly concentrated at their p*I*. It promotes protein aggregation and loss of solubility, and then causes the irreproducibility in protein analysis. The precipitation can be minimized by decreasing the focusing time.

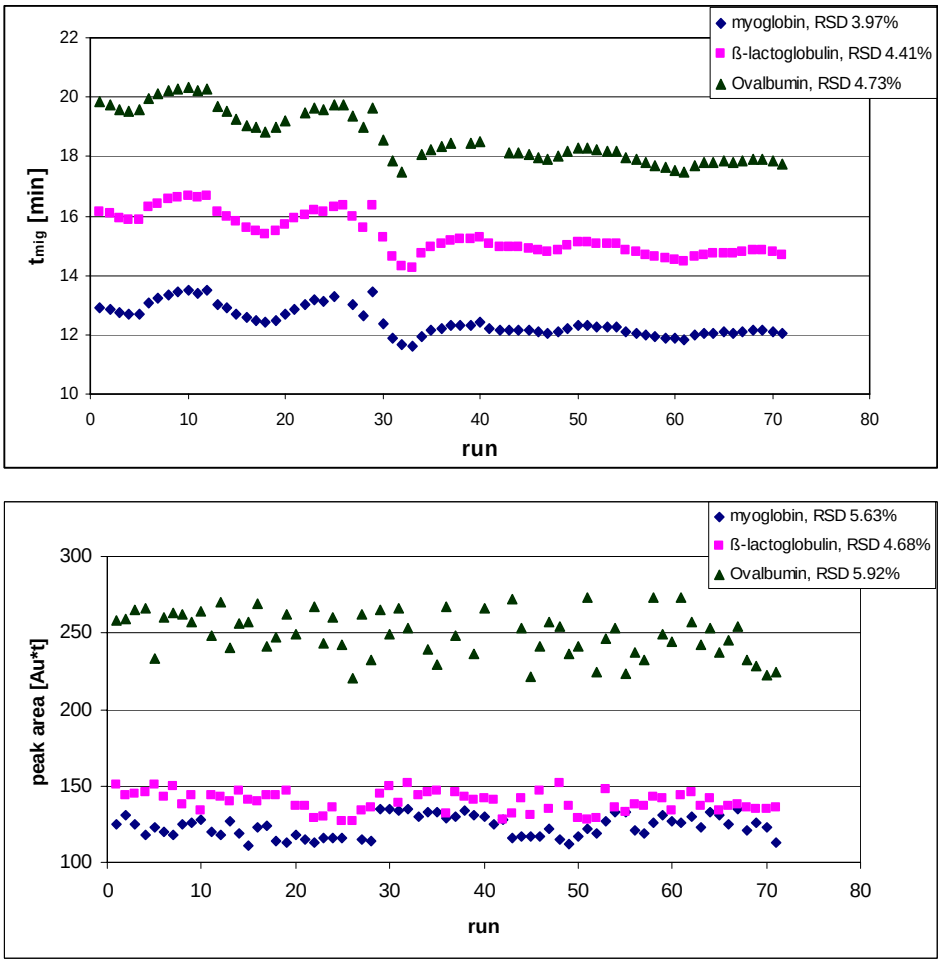


Fig. 43. Precision of protein analysis: migration time and peak area. This series was performed continuously without interruptions during analysis runs. Separation conditions were the same as in Figure 42 using PrinCE 550 CE System. The focusing and mobilization step was performed in the same time at a voltage 30 kV and additional pressure 25 mbar (0.3 – 4.5 µA).

In this experiment, the focusing and mobilization step was performed in the same time at a voltage of 30 kV and by applying a pressure of 25 mbar to reduce the analysis time. Under these conditions, focusing of protein occurs during the mobilization step. As shown in electropherogram at Figure 42, a good compromise between resolution and analysis time was observed even if the focusing step was performed in the same time with the mobilization step. By using this method, the analysis time was also reduced (compare to electropherogram at Figure 36). Consequently, the protein precipitation during analysis can also be reduced. It was shown by the better reproducibility of migration time and peak area of proteins with RSD% values less than 6% in long-term measurements. This result also shows that it is not necessary to wait for complete focusing before the mobilization step starts.

3.2.2.3 The use of internal standards

Based on the investigation of literature, aspects of substance suitability as internal standard in our investigation were considered. Low-molecular-mass substances, such as derivates of amino acids, nicotinic acid, aminobenzoic acid, aminophenylarsonic acid, glutamic acid, anthranilic acid, *etc*. were considered to avoid the precipitation of substances at pH value close to their p*I* [42]. Nevertheless, two substances, namely tryptophan and 4-aminobenzoic acid were selected for further investigations. Both low-molecular-mass substances were chosen according to ampholyte properties, ability of absorption at the wavelength of 280 nm, solubility in water, stability, and non reactivity with sample compounds or ampholytes. As shown in Figure 36, both internal standards showed strong signals, sharp peaks and no overlap with the investigated proteins.

Obviously, there is an additional, still ambiguous migration force for tryptophan apart from pure IEF. Tryptophan showed possibly significant adsorption, e.g. at the amide groups of the polyacrylamide. Adsorption would lead to an increase of migration time and thus pretend a lower value of the p*I*. Usually, adsorptions would cause band broadening and are able to be easily identified. However, when adsorption and IEF focussing occurred at the same time, the band broadening effect will be compensated for by the focussing effect and hence it would be masked. Please note, the observed migration time shift does not impair the good suitability of tryptophan as internal standard.

The precision of protein separation relative to internal standard for long-term measurements with isoelectric focusing was evaluated. As shown in Table 27-30, precision of migration time and peak area of proteins compared to the respective ratios using tryptophan or 4-aminobenzoic acid hardly showed any improvement. The reproducibility of peak area was sometimes found better than peak area ratios and sometimes the other way around. Therefore, the use of internal standard did not significantly improve the results.

As mentioned before, internal standards are able to compensate for injection error, diluting error, sample pre-treatment or solvent evaporation. However, possibly none of these error sources belongs to the most important ones at the present time. Thus, if they compensate for it, the results are not yet improved. As reported by Lacunza, et.al., the lower reproducibility of migration time is probably caused by the proteins providing self carrier ampholyte properties themselves. The proteins thus modify the pH gradient that is established by the ampholyte

[40]. This protein property may change over time, as also found in the previous experiment on protein analysis by CZE (Figure 28 and 29). The resulting change in migration behavior cannot be compensated for by the internal standard that does not show this property. The difficulty on integration of small peak areas also increased RSD% values of peak area [40].

Table 27. Precision on migration time of proteins and relative to internal standard (refer to Figure 41)

Protein	RSD% (n = 130)		
	t_{mig}	t_{mig} / t_{trp}	t_{mig} / t_{abc}
Myoglobin	9.96	11.5	11.9
ß-lactoglobulin	9.05	9.59	10.1
Ovalbumin	8.44	7.44	7.94

Table 28. Precision on peak area of proteins and relative to internal standard (refer to Figure 41)

Protein	RSD% (n = 86)		
	A	A / A_{trp}	A / A_{abc}
Myoglobin	5.09	14.8	6.20
ß-lactoglobulin	8.73	13.2	8.46
Ovalbumin	7.45	14.0	7.08

Table 29. Precision on migration time of proteins and relative to internal standard (refer to Figure 43)

Protein	RSD% (n =71)		
	t_{mig}	t_{mig} / t_{trp}	t_{mig} / t_{abc}
Myoglobin	3.97	2.55	2.39
ß-lactoglobulin	4.41	1.90	1.75
Ovalbumin	4.73	1.39	1.25

Table 30. Precision on peak area of proteins and relative to internal standard (refer to Figure 43)

Protein	RSD% (n = 71)		
	A	A / A_{trp}	A / A_{abc}
Myoglobin	5.63	8.48	7.71
ß-lactoglobulin	4.68	5.82	5.19
Ovalbumin	5.92	6.83	6.40

4. Conclusions

4.1. Protein analysis with capillary zone electrophoresis

The protein separation was studied in capillary zone electrophoresis and isoelectric focusing for preventing protein adsorption on the capillary wall. Some strategies were applied to reduce peak broadening, asymmetric peak shapes, low efficiency, low recovery of analysis, irreversible protein adsorption, a drifting EOF and irreproducible migration times that are caused by protein adsorption.

Deactivation of the silanol groups by coating capillaries is preferable to minimize the wall interactions of protein molecules. The application of PDMAA as a coating for silica offers improved separation efficiency and better reproducibility of the EOF mobility compared to bare fused-silica capillaries, especially at a pH close to the p*I* of the protein. The stability of the PDMAA coating was also achieved in long-term protein separation. Even though the PDMAA-coated capillaries can minimize the protein-wall interaction, it cannot prevent it completely.

A less stable protein can undergo conformational changes to denatured states after protein adsorption. Trehalose and sucrose have been demonstrated for keeping stability of the native state of a protein in solution during the separation process in the literature. However, in general, no significant difference in the reproducibility of EOF mobility was observed between the presence and the absence of trehalose and sucrose during protein analysis. This indicates that the suggested influence of trehalose and sucrose on reducing protein adsorption could not be confirmed.

Poly(ethylene glycol) was proven as an effective substance to stabilize the proteins native state and coat the bare fused-silica capillary surface. The presence of 32 mg/mL PEG in protein and buffer solution in a range of pH 6.0 to 4.0 was successful to suppress protein adsorption during the separation. It can also be confirmed with the reproducibility of apparent EOF mobility with percental RSD less than 2% in long-term measurement.

The regeneration of the capillary with the rinsing reagent is important for avoiding ageing effects. 2M hydrochloric acid was proven as a reliable rinsing reagent to remove adsorbed proteins on linear polyacrylamide-coated capillaries. Phosphoric acid 85% (m/m) was even more effective for especially protein samples with high concentration or the more easily

defolding one and therefore stronger in adsorbing ß-casein. Good precision in long series which lasted approximately one week each was also observed. There was no evidence that the capillaries changed during these series. Possibly one capillary can be used even much longer. In general, this procedure is quite simple to use and significantly improve the precision of protein analysis by CE.

4.2. Protein analysis with capillary isoelectric focusing

Using rinsing procedures, proteins were reproducibly focused and separated on linear polyacrylamide-coated capillaries by isoelectric focusing at a pH gradient 3-10. In order to avoid capillary blockage caused by protein adsorption on linear polyacrylamide-coated capillaries, 3M hydrochloric acid could be used as a rinsing reagent after each run. Reliable CIEF in long series was achieved for the first time. With the purpose of improving the reproducibility of migration time and peak area, rinsing procedures during capillary storage could also be performed. The rinsing with water in the company of applying pressure and high voltage is able to prevent assumed bulged structure on the coated capillaries. In this case, an improved RSD% value was observed. However, consecutive runs without capillary storage during routine analysis provide further improvement in reproducibility of migration time and peak area with RSD% values less than 10% in a long-term measurements. Decreasing the focusing time can also reduce the protein precipitation during analysis. It offers better reproducibility of migration time and peak area with RSD% values less than 6%.

At the present time, tryptophan and 4-amino-benzoic acid as internal standard are not suitable to improve the precision of protein separations by isoelectric focusing. In general, a good resolution of protein analysis was observed with capillary isoelectric focusing. With the rinsing procedure described above, better precision of migration time and peak area was noticeably observed. Nevertheless, the precision of the procedure is inferior to the generally reported precision using capillary zone electrophoresis.

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